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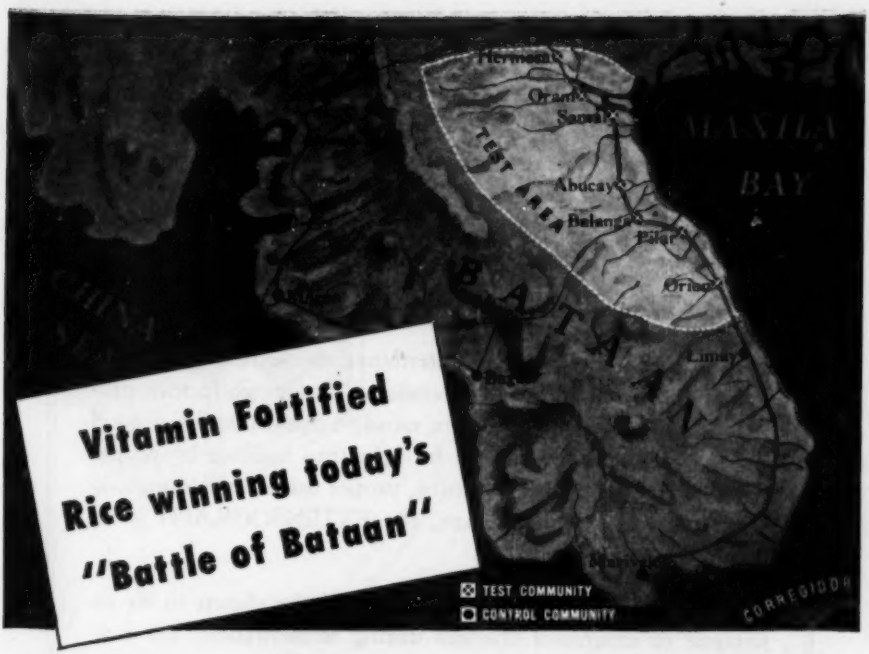

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*Quotation from latest of published interim medical reports. *Write for reprint.*

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STUDY OF PROTEOLYTIC ACTIVITY IN WHEAT FLOUR DOUGHS AND SUSPENSIONS. II. A PAPAIN INHIBITOR IN FLOUR[1,2]

B. D. HITES, R. M. SANDSTEDT AND LORENE SCHAUMBURG

ABSTRACT

Wheat flour contains an inhibitor that retards the action of papain and ficin, but which, in so far as is presently known, has no effect on the native proteolytic enzymes of flour. It is water soluble and non-dialyzable and may be concentrated by freeze-drying the water extracts. The papain inhibiting action is not affected by relatively large quantities of sulfhydryl compounds; smaller amounts of oxidizing agents such as bromates, chlorites, iodates, and persulfates increase the amount of papain inhibited probably due to their own well-known action on papain. The non-dialyzable portion of dialyzed extracts inhibit greater quantities of papain than do the undialyzed extracts. The inhibitor has been found in all grades of flour and in higher concentration in wheat bran. Proteolysis in fermenting doughs containing papain was retarded by the presence of sufficient quantities of the inhibitor. The inhibitor free from the native enzymes was prepared—the treatment for destroying the native enzymes increased the effectiveness of the inhibitor. Reaction curves show the retarding effects of the inhibitor on the action of papain when present in suspensions: of flour, of flour bacto-hemoglobin mixtures, and of bacto-hemoglobin alone.

Proteolytic enzymes seemingly are present in normal wheat flour doughs in exceedingly small amounts or for some reason are quite inactive on flour proteins. Balls and Hale (5) state, "The chemical changes in flour that can be attributed to the action of the native protease appear to be very small as measured by any of our present day methods. On the other hand, changes to be observed in the physical condition of the dough are very striking." There is little information concerning the kinetics of the action of the native enzymes of flour other than that obtained by physical methods. Since there is considerable doubt whether changes in physical characteristics may be considered as evi-

[1] Manuscript received June 15, 1950; Presented at Annual Meeting, May, 1950. Published with the approval of the Director as paper No. 505, Journal Series, Nebraska Agricultural Experiment Station.

[2] Contribution from the Department of Agricultural Chemistry, University of Nebraska, Lincoln, Nebraska.

dence of proteolysis this situation has led many to question the importance of proteolysis in doughs. The literature concerning proteolysis in doughs and the action of oxidizing and reducing agents has been adequately reviewed by Hildebrand (8).

Since the kinetics of the action of the native proteolytic enzymes of flour are still largely unknown and since the mode of action of oxidizing and reducing agents is still controversial, it was thought advisable to study the action on doughs of some similar protease which could be added in definite quantity. The action of such an enzyme could then be used as a model for a further study of the naturally occurring proteolytic enzymes. Papain was chosen because the wheat flour proteases have been characterized as papainases (3), i.e., they are said to be of the papain type and accordingly must be activated by certain reducing agents and inhibited by some oxidizing agents. This characterization is the basis for the Jørgensen (9) and Balls and Hale (4) explanations for the improving action of oxidizing agents on bread doughs.

Materials and Methods

The Ayre-Anderson method for the determination of proteolysis (2) with its modifications (10) (11) was used in this study. The method as used involved the autolytic digestion at 40°C. of 10 g. of flour with 52 ml. of solution containing 2.5 ml. of 4 *M* acetate buffer, pH 4.7.[3] In case hemoglobin (1) was used as a supplemental substrate in addition to the flour, 1.25 g. was mixed with the 10 g. of flour before adding the liquids, or in case the hemoglobin was the entire substrate, the 1.25 g. of hemoglobin replaced the 10 g. of flour.

At the end of the digestion period the suspension was clarified by thoroughly mixing with 4 ml. of 45% sodium tungstate followed by 12 ml. of 5 *M* trichloroacetic acid.[4] The precipitation was followed by centrifuging at 11,000 rpm. (15,000 × G) for 5 minutes with subsequent decantation through a No. 4 Whatman filter paper. Aliquots of from 25 ml. to 40 ml., depending on the nitrogen present, were taken for Kjeldahl determinations. The nitrogen found was calculated to total non-precipitable nitrogen (NPN) per 10 g. of flour and this figure was then corrected to represent the NPN produced by enzyme action on 10 g. of flour. Suitable control tests were made for all periods of time covered by the particular experiment.

The baking method employed in this study was the micro-baking technique used by Sandstedt and Fortmann (12). The papain was a

[3] The acetate buffer was satisfactorily effective as a preservative. The use of toluol, octyl alcohol, dehydroacetic acid, and Roccal should be avoided as they have marked effects on proteolysis under certain conditions.

[4] It was not always possible to obtain clear filtrates by clarification with trichloroacetic acid alone. Cloudy filtrates indicate incomplete precipitation which may cause discrepencies due to variability in rates of adding the precipitating agent, rates of stirring, centrifugal speeds, and filtering techniques. Water clear filtrates were obtained with the sodium tungstate-trichloroacetic acid procedure

standardized sample obtained in 1945 from Merck and Company. Three commercially milled unbleached hard winter wheat flours were used: an 85% patent, containing 11.3% protein and 0.43% ash, a first clear with 12.9% protein and 0.61% ash, and a second clear, with 15.1% protein and 0.86% ash (14% moisture basis).

Extracts of these flours were made by mixing 1 part of flour by weight with 2 parts of water in a Waring blendor for 5 minutes followed by centrifugation. Some of these water extracts were dialyzed and others were not dialyzed before freeze-drying. For comparative purposes it was assumed that, volume for volume, each milliliter of liquid remaining in the residue would hold as much dissolved material as each milliliter of the supernatant. Thus the flour equivalent of any aliquot of extract was calculated on this basis; e.g., 20 ml. of a 1:2 flour extract was considered an equivalent of 10 g. of flour. The hemoglobin was Difco Bacto-hemolgobin.

Results and Discussion

Figure 1 shows curves that represent the NPN produced in suspensions of flour and of flour-hemoglobin mixtures which had autolyzed for increasing lengths of time at 40°C. Little NPN was produced in the suspension of flour alone over a six hour period (curve 1) whereas considerable NPN was produced in the flour plus bacto-hemoglobin substrate during the same period (curve 2). Apparently the wheat flour contained proteases which, under these conditions had only a limited action on flour protein. Bergmann (6) states that

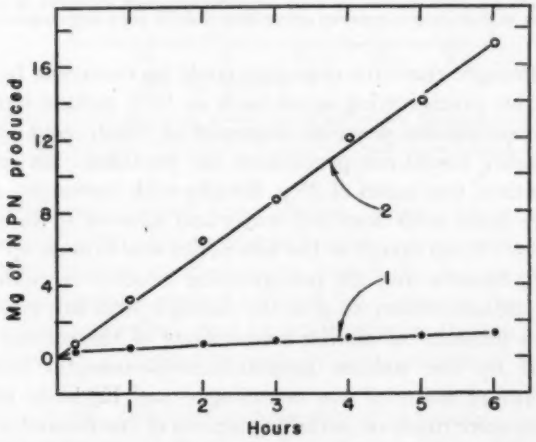

FIG. 1. The action of the native flour proteases on flour proteins as compared to their action on flour plus bacto-hemoglobin. Suspensions of unbleached patent flour buffered to pH 4.7 and digested at 40°C. for increasing lengths of time: 1, Flour alone; 2, Flour plus bacto-hemoglobin.

enzymes require not only specific backbone groups in the protein substrate but specific side chain groups also before they are able to attack. Possibly the resistance of the flour proteins to digestion by the proteases of flour may be explained in some such way.

The Ayre-Anderson method is based on the determination of non-precipitable nitrogen as an index of proteolysis. There has been objection to the use of such methods because proteolytic action may bring about the cleavage of protein molecules into large peptide chains which themselves would be removed by such precipitating agents as phosphotungstic acid, trichloroacetic acid, or a combination of sodium tungstate and trichloroacetic acid and, therefore, would not be determined in the supernatant substrate as proteolytic decomposition products.

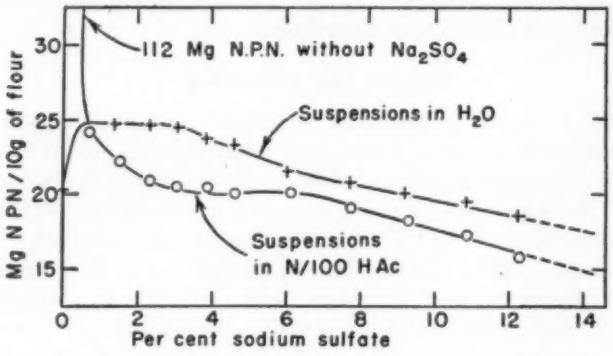

FIG. 2. Comparison of NPN obtained by using increasing increments of Na_2SO_4 as the precipitating agent on flour-water suspensions and on flour—0.01 *N* acetic acid suspensions.

It was thought that this objection could be overcome by the use of a less effective precipitating agent such as 10% sodium sulfate which effectively precipitates proteins dispersed in dilute acid (Fig. 2) but which probably would not precipitate the peptides. In order to test this supposition, two series of 25 g. doughs with increasing amounts of papain were made with flour and water and allowed to digest for four-hour periods. Each dough of the first series was broken up with water in a Waring blendor and the precipitation effected by adding enough anhydrous sodium sulfate to give the liquid a 10% salt concentration. The protein material of similar suspensions of the second series was precipitated by the sodium tungstate-trichloroacetic acid method. The precipitated material was centrifuged and Kjeldahl nitrogen determinations were made on suitable aliquots of the filtered supernatant liquids. The curves in Fig. 3 show that although much more non-precipitated nitrogen was found in the extracts clarified with sodium

sulfate, both methods indicate that no proteolysis had taken place until about 6 mg. % of papain was added but that additional papain caused a rapid increase. When the curves are corrected so as to represent the non-precipitated nitrogen produced by papain action (by subtracting the non-precipitable nitrogen of the untreated suspension) as shown in curves 3 and 4 of the figure, it is observed there is a divergence of the curves (3 and 4) at the 6 mg.% papain level, presumably the point at which proteolysis begins. This indicates that there were peptides formed by papain action which were not precipitated by the sodium sulfate but which were thrown out of solution by the sodium

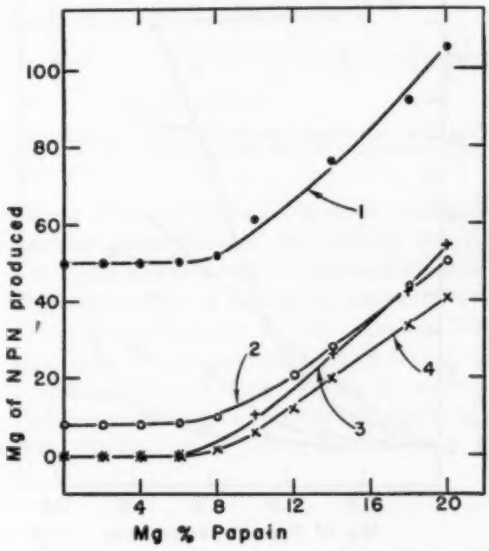

FIG. 3. Comparison of precipitation by sodium sulfate and sodium tungstate-trichloroacetic acid. Four hour yeastless doughs. NPN found in extract: 1, After precipitation with 10% Na_2SO_4; 2, After precipitation with tungstate-trichloroacetic acid. Curves 3 and 4 are curves 1 and 2 corrected to give NPN produced by papain action.

tungstate-trichloroacetic acid precipitating agent. However, not all of the proteolytic degradation products were precipitated. Accordingly both precipitation methods gave essentially the same information, i.e., that proteolysis started at the 6 mg.% papain concentration. The sodium sulfate method may be preferable in studies where it is applicable, however, it cannot be used for precipitation of hemoglobin suspensions.

The curves of Fig. 4 illustrate the action of increasing quantities of papain on suspensions of flour, of flour-hemolgobin, of hemoglobin alone, and of hemoglobin plus the dried non-dialyzable extract from 30

g. of flour. Similar to the data presented in Fig. 3, curve 3 of Fig. 4 shows that the first 0.6 mg. of papain had very slight action on the 10 g. of flour but that when the papain content was greater than 0.6 mg. there was a rapid increase in activity. Action on hemoglobin began with the first increments of papain (curve 2), but the presence of flour in the hemoglobin substrate (curve 1) decreased the action of the papain on the hemoglobin as shown by the first part of the curve. The rapid action shown in curve 1 on the addition of more than 0.6 mg. of papain is due to papain action on both hemoglobin and on flour

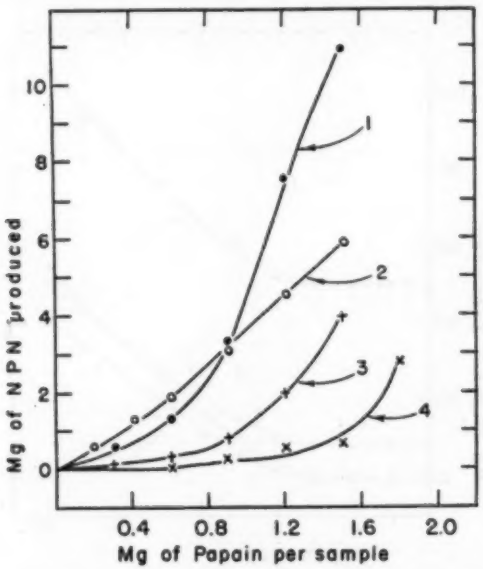

FIG. 4. The action of increasing amounts of papain on the substrates: 1, Patent flour + hemoglobin; 2, Hemoglobin alone; 3, Patent flour alone; 4, Hemoglobin + dialyzed and dried extract from 30 g. (3X) of patent flour. The NPN of the original flour and that produced by the action of the native flour proteases has been subtracted from the original data to give the data shown.

protein. Curve 4 indicates that papain attacked bacto-hemoglobin in the presence of flour extract (equivalent to 30 g. of flour) very slowly until more than 1.5 mg. of papain had been added. These data suggest that the wheat flour contained an inhibitor which effectively protected the flour proteins from the action of small amounts of papain. However, the flour proteins were susceptible to the action of papain as is evidenced by the action of quantities of papain above the 0.6 mg. level (6 mg.% based on the flour). This inhibitor was capable also of protecting hemoglobin from papain attack (compare curves 1 and 4 with curve 2).

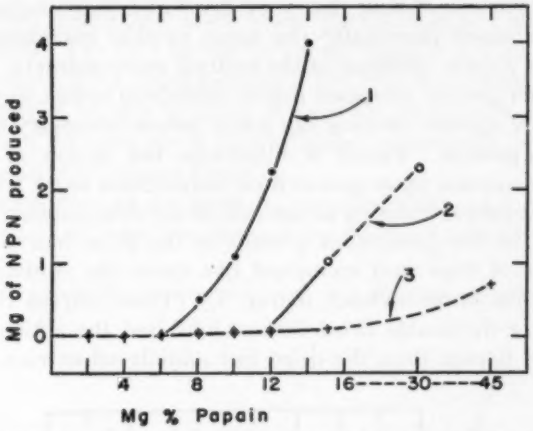

FIG. 5. The inhibiting effect of flour solubles on papain action. Four hour digests of patent flour at pH of 4.7 and 40° C.: 1, Flour alone; 2, Flour plus the dried extract from 10 g. (X) of flour; 3, Flour + 5X of dried extract.

The inhibiting effect of the soluble flour constituents on papain action was further demonstrated by adding dried water solubles equivalent to as much as five times that already present in the flour and then determining the action of increasing amounts of papain on these substrates. The data are shown by the curves in Fig. 5. The presence of an extra equivalent of flour solubles extended the papain inhibition from 0.6 mg. to about 1.2 mg. (curve 2), whereas five times as much of the solubles inhibited more than five times as much papain as was inhibited by the flour itself (curve 3).

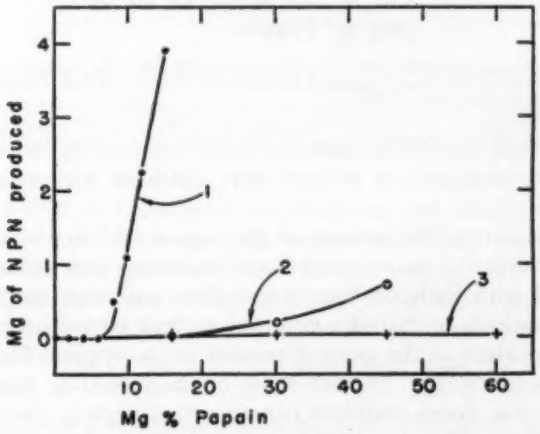

FIG. 6. The increased effectiveness of the dialyzed inhibitor. Four hour digests of: 1, Patent flour only; 2, Patent flour + 5X of undialyzed dried extract; 3, Patent flour + 5X of dialyzed dried extract.

Water extracts of flour that were dried at low pressures by freeze-drying contained practically the same (within experimental error) quantity of papain inhibitor as the undried water extracts. However, they showed greatly increased papain inhibiting action when dialyzed for 48 hours against running tap water before freeze-drying the non-dialyzable portion. Figure 6 illustrates the action of increasing amounts of papain upon patent flour suspensions alone (curve 1), on flour suspensions containing an amount of the dried undialyzed extract equivalent to five times that present in the flour (curve 2) and on suspensions of flour that contained five times the equivalent of the dried non-dialyzable extract (curve 3). These curves demonstrate that the non-dialyzable dried extract inhibited the action of greater amounts of papain than the dried but undialyzed extract. It is hy-

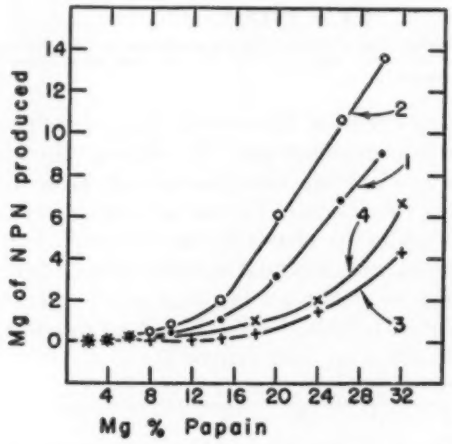

FIG. 7. The papain inhibiting action of unbleached clear flours. Four hour suspensions: 1, First clear; 2, Second clear; 3, First clear + undialyzed dried extract from 10 g. of flour; 4, Second clear + undialyzed dried extract from 10 g. of flour.

pothesized that dialysis denatured some of the proteins (or some of the native flour protease) to release more inhibitor for action against papain.

To demonstrate the presence of the papain inhibitor in unbleached commercial first and second clear flours, increasing quantities of papain were mixed with buffered flour suspensions and with suspensions of these flours which contained a quantity of their own dried undialyzed extract equivalent to the amount present in the original flour. Comparing these data (Fig. 7) with those of the preceding figures shows that these clear flours inhibited papain action slightly more than did the patent flour. The slopes of the curves indicate that the papain ac-

tivity increased more slowly after action started than was observed for the patent flour. The data shown by curves 3 and 4 of Fig. 7 indicate that the presence of the dried extracts in the flour suspensions required larger amounts of papain to cause perceptible proteolysis, and hence further confirm the presence of papain inhibiting material in the water soluble extracts of these two flours.

Since papain may be activated by certain reducing agents, including glutathione and may be inhibited by oxidizing agents, it was of

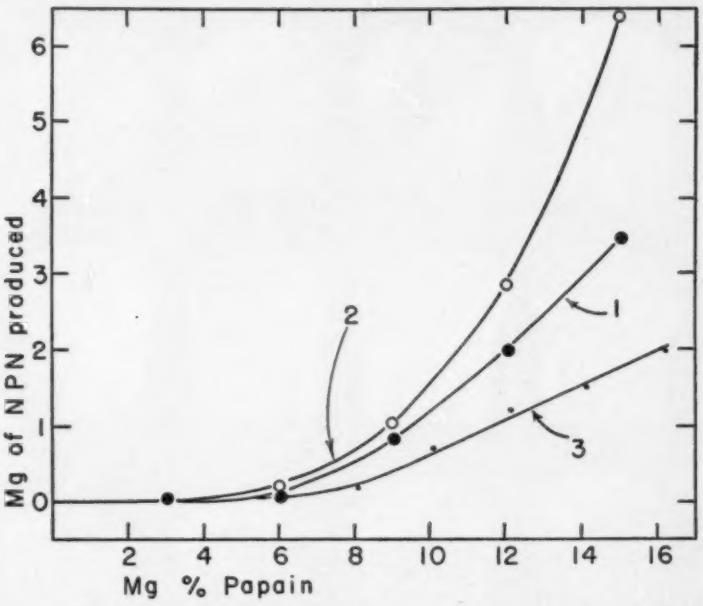

FIG. 8. The effects of glutathione and chlorite on papain action and on papain inhibition. Unbleached patent flour, suspended in buffer at pH of 4.7 for 4 hours at 40°C.: 1, Action of increasing increments of papain on flour alone; 2, On flour with 15 mg. % of glutathione; 3. On flour with 3 mg. % of sodium chlorite.

interest to determine the effects of glutathione and chlorite on the action of papain in the presence of the papain inhibitor. Figure 8 compares the action of papain on flour with and without the addition of 15 mg.% (flour basis) of glutathione and of 3 mg.% of chlorite. The glutathione was unable to activate the inhibited papain (the first portion of curve 2 falls on curve 1) but was effective on the uninhibited papain (divergence of curves 1 and 2). The chlorite effectively increased the amount of papain inhibited which may indicate that the inhibiting actions of the two agents were additive.

Papain Inhibitor Effects Shown in Doughs and Bread. The data obtained by determinations of non-protein nitrogen given above show that the presence of a papin inhibitor in flour may be demonstrated by chemical means. If these data are significant as far as baking is concerned, it should be possible to bake bread from yeasted doughs to which increasing amounts of papain have been added at the mixing stage and demonstrate, both by the handling properties of the doughs and by the character of the baked bread, that the papain has little

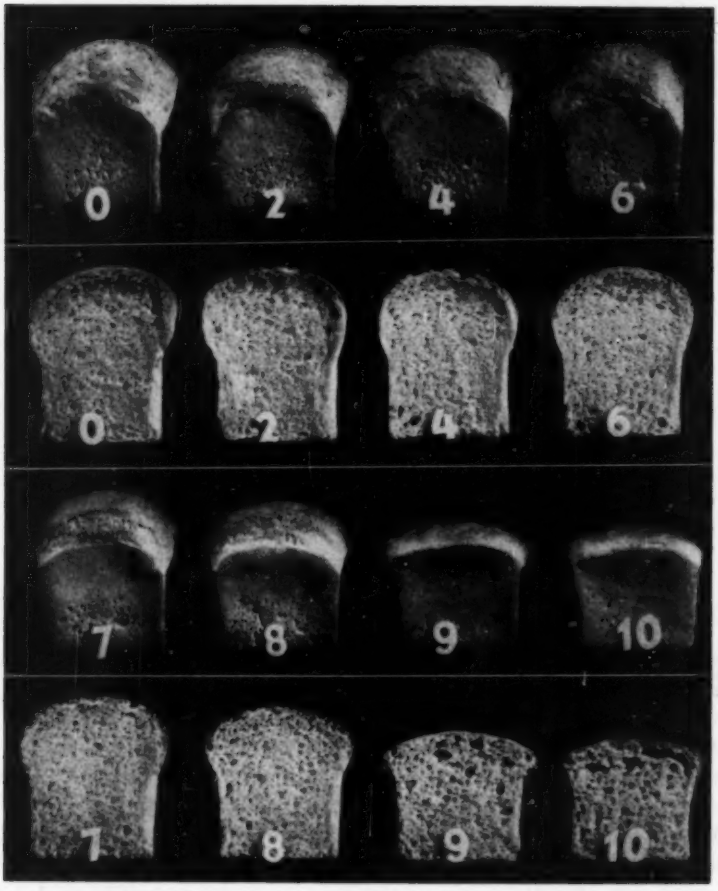

FIG. 9. Bread baked from a series of doughs which contained progressively increasing quantities of papain. The numbers on the loaves denote the mg% amounts of papain present in the doughs.

0—loaf volume 160 cc.
2—loaf volume 159 cc.
4—loaf volume 151 cc.
6—loaf volume 149 cc.
7—loaf volume 142 cc.
8—loaf volume 133 cc.
9—loaf volume 117 cc.
10—loaf volume 110 cc.

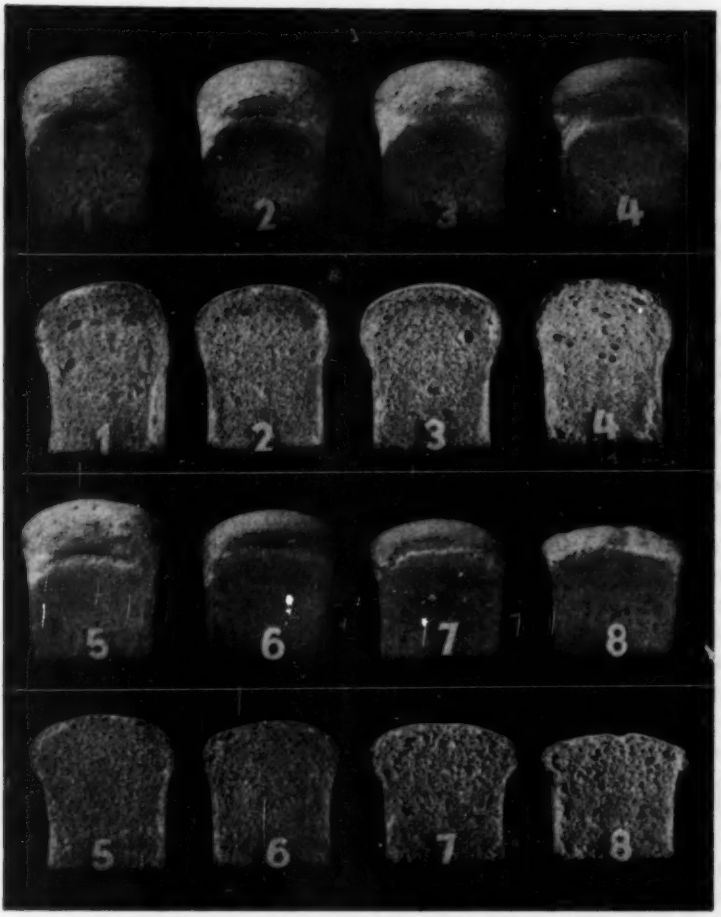

FIG. 10. Effect of added inhibitor on loaves baked from papain treated doughs.

Loaf 1—Check, no inhibitor, no papain—volume 157 cc.
Loaf 2—2X inhibitor, no papain—volume 156 cc.
Loaf 3—2X inhibitor, 9 mg.% papain—volume 150 cc.
Loaf 4—2X inhibitor, 12 mg.% papain—volume 146 cc.
Loaf 5—2X inhibitor, 15 mg.% papain—volume 136 cc.
Loaf 6—2X inhibitor, 18 mg.% papain—volume 125 cc.
Loaf 7—2X inhibitor, 21 mg.% papain—volume 122 cc.
Loaf 8—2X inhibitor, 24 mg.% papain—volume 114 cc.

effect on the doughs or bread until enough has been added to exceed the inhibitor value.

Micro-doughs containing from 0 to 10 mg.% of papain (flour basis) were made from a commercial unbleached first clear flour. All doughs were proofed to height where possible. The doughs with 7 mg.% of papain or less handled normally but with greater quantities they be-

came increasingly sticky and hard to handle. The baked loaves are shown in Fig. 9. The loaf volume decreased very slowly as the papain was increased until 9 mg.% had been added when there was a marked decrease in the size of the loaf. Apparently in this loaf the papain was in excess of the inhibitor, leaving an excess of the enzyme to act on the flour proteins. With 10 mg.% of papain, the doughs became thin and no longer proofed to height but tended to run over the sides of the pans. The loaves, both internally and externally, began to appear "young" or underdeveloped when the papain exceeded 6 mg.%.

Further proof of the papain inhibiting action of flour solubles was obtained by adding 2 equivalents of non-dialyzable dried extract to a series of doughs in which papain varying from 9 mg.% to 24 mg.% was mixed in increasing increments of 3 mg.% for each succeeding dough. A comparison of loaves 1 and 2 of Fig. 10 shows that the non-dialyzable inhibitor had little effect on the loaf volume and crumb. The doughs at panning time were not too slack or sticky to handle except the one containing 24 mg.% of papain (loaf 8). It did not proof to height and had no oven spring. The loaves took on a "younger" appearance beginning with loaf No. 5 which contained 15 mg.% of papain compared to 7 mg.% in the loaves with no extra inhibitor (Fig. 9). The crumb began having a reduced or underdeveloped appearance also in loaf 5. Note that the data of Fig. 9 indicate that 10 mg.% of papain was required to completely destroy the baking properties of the doughs with normal inhibitor content, whereas more than 24 mg.% was needed to bring about the same degree of apparent proteolysis in the doughs containing the additional 2X inhibitor. These two sets of photographs give additional evidence of the presence of an effective papain inhibitor in flour.

The effectiveness of the inhibitor in eliminating the action of small quantities of papain was further demonstrated by adding 5 mg.% (flour basis) of papain to buffered flour suspensions and to yeastless doughs of unbleached patent flour and allowing them to stand (the doughs at 30° and the suspensions at 40°) for increasing lengths of time up to sixteen hours. At each time interval the papain treated doughs displayed essentially the same handling properties as doughs that were papain free. The curves given in Fig. 11 show the action of the 5 mg.% of papain in the flour suspensions during the 16 hours digestion. Only a small increase in NPN (1 mg. per 10 g. of flour in 16 hours) can be attributed to the papain action. Apparently this quantity (5 mg.%) of papain was effectively inhibited by the normal inhibitor of the flour.

It should be emphasized that the quantitative relationships obtained in this research may not be obtained using different flours or different preparations of papain. Hale (7) states that, "One part of

commercial papain to twenty thousand parts of flour (5 mg.%) may completely liquefy a dough." Sandstedt and Fortmann (12) obtained drastic proteolysis in fermenting doughs with 3 mg.% of papain. Flours may quite probably vary widely in inhibitor content (although this has not been shown to be true in the flours so far tested) and commercial preparations of papain also may vary in activity; one non-standardized preparation of papain recently received was nearly 10 times as active as the other preparations on hand. Using this preparation, the normal inhibitor of a patent flour only inhibited 0.6 mg.%.

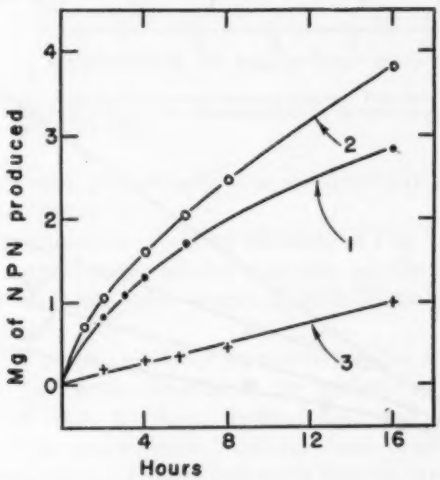

FIG. 11. The action of 5 mg.% of papain over a 16 hour digestion period: 1, Curve 1 shows NPN produced in flour suspensions with no papain; 2, The NPN produced in flour suspensions containing 5 mg.% of papain; 3, The amounts of NPN produced by papain action (Curve 2 minus curve 1).

Action of the Inhibitor on Greater Quantities of Papain During a Long Digestion Period. It was estimated from curves 1 and 2 of Fig. 7 that for 4 hour digestion periods the inhibitor retarded the action of about 7 mg.% of commercial papain. When an amount of the dried inhibitor preparation equivalent to that contained in 10 g. of flour was added to the flour suspensions (thus doubling the amount of inhibitor) in the presence of 14 mg.% of papain the action of the papain was greatly retarded when compared to suspensions containing papain and no added inhibitor. The curves of Fig. 12 show the retarding effect of the inhibitor on the action of 14 mg.% of papain over a 16-hour digestion period. It was thought that some of the proteolytic action displayed during the first two or three hours (curve 2) may have been due to the papain acting on the flour proteins before a complete reaction between the enzyme and the inhibitor was attained. Curve 4 shows

the increased inhibition obtained by allowing the papain and inhibitor to stand together in solution for four hours prior to mixing them with the flour suspension. This treatment increased the effectiveness of the inhibitor.

The Action of the Inhibitor on the Native Four Proteases. The data presented in Fig. 1 indicate that the native flour-proteases are active on hemoglobin but that they have little action on the flour proteins. May this be explained by the presence of the proteolytic inhibitor in

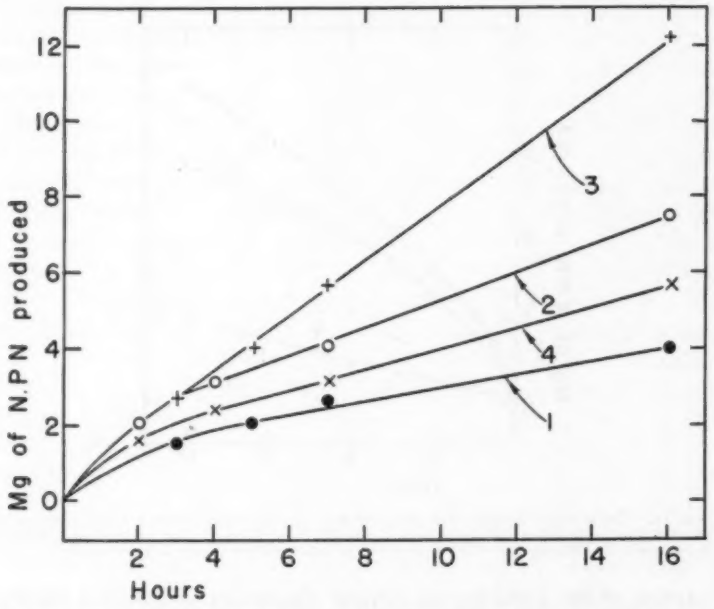

FIG. 12. The effects of the inhibitor on long time digestions of flour with 14 mg.% of papain: Curve 1, flour suspensions without papain; Curve 2, flour suspensions plus 14 mg.% of papain and X of dried flour extract; Curve 3, flour suspensions plus 14 mg.% papain with no added inhibitor (extract); Curve 4, suspensions to which 14 mg.% papain and X dried extract were added after the latter two had stood together in buffer for a 4 hour period prior to mixing with the flour.

the flour? To obtain evidence on this question increasing increments of dried flour extract were added to flour suspensions and to hemoglobin suspensions. The data are shown in Fig. 13. Increasing amounts of dried extract produced linear increases in proteolytic activity on the hemoglobin (curve 2) but no increases in NPN were found for corresponding additions of the extract to the flour substrate. This dried extract was the same as that used for obtaining the data presented in Fig. 5 which showed that this extract was a papain inhibitor. However, the papain inhibitor did not prevent the native

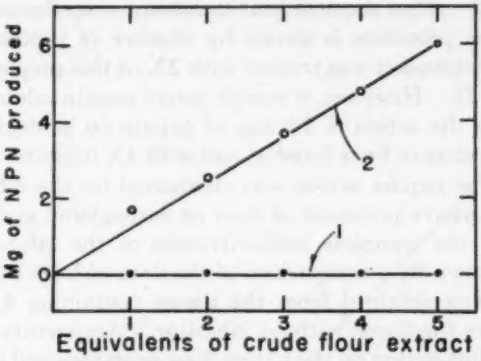

FIG. 13. The action of the inhibitor on the native proteases. Four hour digestions of increasing quantities of dried flour extract on: 1, Flour suspensions; 2, Bacto-hemoglobin suspensions. One equivalent = extract from 10 g. of flour.

enzymes, which were present with it in the dried extract, from attacking hemoglobin (curve 2).

Some of the questions raised by the data of Fig. 13 concerning the action of the native water soluble enzymes on the proteins of flour (curve 1) and on hemoglobin (curve 2) will be discussed in another paper of this series.

The inability of the inhibitor to inactivate the native proteolytic enzymes may be better illustrated by adding large quantities of enzyme-free inhibitor to flour, hemoglobin, and flour-hemoglobin suspensions. The enzyme-free inhibitor was prepared by adding enough acetic acid to a 1:2 water extract of flour to lower the pH to 5.0, heating the solution at 80°C. for 3 minutes, dialyzing against tap water, centrifuging, and freeze-drying the supernatent. Table I gives the

TABLE I

THE ACTION OF ENZYME-FREE INHIBITOR ON PAPAIN AND ON NATIVE FLOUR ENZYMES

No.	Substrates	Digestion Time[1] Min.	Mg. Total NPN	Mg. NPN Due to Proteolysis
1	Hb + 2X inhibitor	15	2.4	
2	Hb + 2X inhibitor	240	2.4	0.0
3	Hb + 1.5 mg. of papain	15	2.4	
4	Hb + 1.5 mg. of papain	240	8.2	5.8
5	Hb + 4X inhibitor + 1.5 mg./papain	15	3.1	
6	Hb + 4X inhibitor + 1.5 mg./papain	240	3.2	0.1
7	Hb + flour	15	6.0	
8	Hb + flour	240	19.1	13.1
9	Hb + flour + 4X inhibitor	15	9.5	
10	Hb + flour + 4X inhibitor	240	22.3	12.8

[1] Digestion Temperature = 40°C.

data obtained. That the acid heat treatment was effective in destroying the native proteases is shown by absence of proteolysis when a hemoglobin suspension was treated with 2X of this preparation (Table I, lines 1 and 2). However, it was an active papain inhibitor as shown by comparing the action of 1.5 mg. of papain on hemoglobin without inhibitor (the data of lines 3 and 4) and with 4X inhibitor (data of lines 5 and 6). The papain action was eliminated by the inhibitor. The action of the native proteases of flour on hemoglobin is shown in lines 7 and 8 and the complete ineffectiveness of the inhibitor on these enzymes is shown by a comparison of the data of lines 8 and 10. The same NPN was obtained from the digest containing 4X heated inhibitor as from the digest without inhibitor. Apparently the inhibitor had no retarding action on the native flour proteases and accordingly it is probably not the factor responsible for the resistance of the flour proteins to native enzyme action.

Further evidence that the enzyme-free extract is an effective papain inhibitor is presented in Fig. 14. It seems that destroying the native enzymes (or denaturation of other proteins) increased the effectiveness of the inhibitor.

That wheat bran also contains the papain inhibitor but in greater quantities than is found in flour is shown by the data of Fig. 15. The dried extract (undialyzed) from 10 g. of bran extended the inhibition from approximately 6 mg.% papain to 20 mg.% whereas the extract from flour (Fig. 5) extended the inhibition from 6 mg.% to about 12 mg.%.

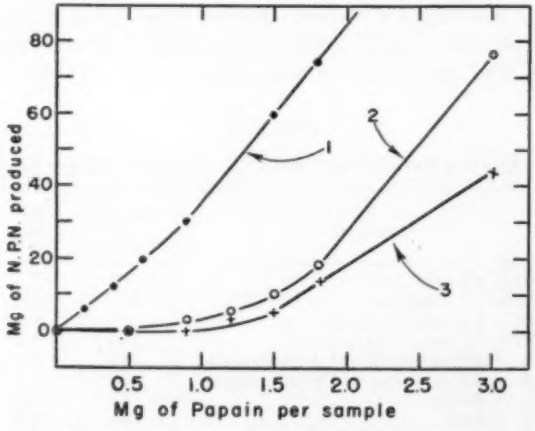

FIG. 14. Comparison of the inhibiting action of flour extract with that of enzyme-free extract. Action of papain on hemoglobin: 1, With no inhibitor; 2, With 3X of non-dialyzable dried extract; 3, With 3X of enzyme-free non-dialyzable dried extract. Extract from 10 g. of flour. The action of the native proteolytic enzymes was removed from the data plotted in curve 2 by subtracting the NPN found in a mixture of hemoglobin and extract (no papain) at the end of the 4 hour digestion.

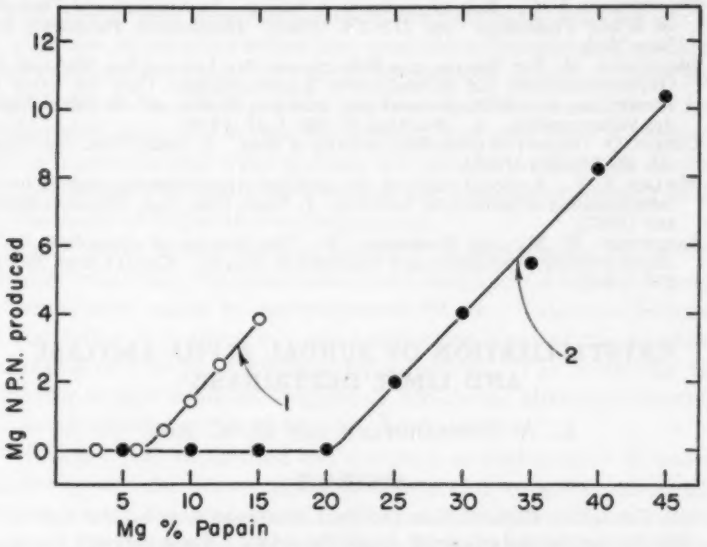

FIG. 15. The inhibiting action of wheat bran extracts. Action of papain: 1, On flour; 2, On flour + the undialyzed dried extract from 10 g. of bran.

The above data show the presence in flour of a papain inhibitor, which may be a papainase inhibitor. Some differences between the action of papain and the action of the native proteases of flour are indicated. These differences raise considerable doubt regarding the assumption that data obtained concerning the action of papain on flour may be considered as evidence of the action of the flour proteases. The water soluble native proteases were active on hemoglobin and this action was not affected by the papain inhibitor. However, these soluble enzymes caused no proteolysis of the flour proteins.

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CRYSTALLIZATION OF FUNGAL ALPHA-AMYLASE AND LIMIT DEXTRINASE [1]

L. A. UNDERKOFLER and D. K. ROY [2]

ABSTRACT

Crystalline alpha-amylase and limit dextrinase were isolated from a filtrate of submerged culture of *Aspergillus oryzae*. These enzymes, free of maltase, were concentrated together by repeated ammonium sulfate precipitations, with a Bentonite adsorption interspersed after the first precipitation. The first crystalline precipitate obtained showed two distinctly different types of crystals.

To obtain crystalline alpha-amylase, the ammonium sulfate precipitate was dissolved in potassium phosphate buffer at pH 4.6 and the limit dextrinase adsorbed on Bentonite. The filtrate from this treatment yielded hexagonal pyramid shaped crystals of alpha-amylase upon the addition of ammonium sulfate. A solution of these crystals showed no limit dextrinase activity.

To obtain limit dextrinase, the ammonium sulfate precipitate was dissolved in distilled water, dialyzed, and the solution treated with mercuric chloride. This treatment destroyed alpha-amylase with only slight deleterious effect on limit dextrinase. The solution was dialyzed, precipitated with ammonium sulfate, and the precipitate recrystallized by dissolving in water and adding ammonium sulfate and sodium chloride. The crystals of limit dextrinase were long, shiny, silky needles. A solution of these crystals showed a barely detectable trace of alpha-amylase activity and extremely high limit dextrinase activity.

The mold *Aspergillus oryzae* has long been cultivated for the production of enzymes, particularly starch-hydrolyzing enzymes. Commercial amylase concentrates from this organism are well known and are employed for many important industrial uses. Recently considerable interest has developed among cereal chemists in the possible use of such enzyme concentrates for diastatic fortification of flours.

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It has long been recognized that in addition to the starch-hydrolyzing enzymes, *Aspergillus oryzae* also produces a considerable number of other enzymes. Tauber (22) listed 23 different enzymes which have been reported for this organism. Likewise, it has been known for a considerable time that starch-hydrolyzing enzymes from *A. oryzae* as well as from malt and other sources, are enzyme complexes. Redfern (19) has recently reviewed developments in amylase chemistry including the mode of action of the components.

Early study of the kinetics of starch hydrolysis by barley malt enzymes resulted in the observation that starch is not wholly degraded to fermentable sugars by malt enzymes (9, 6). Euler and Svanberg (4) concluded that there is a saccharification limit of the process of hydrolysis of starch to maltose by amylases. The relatively high molecular weight residual compounds remaining after malt saccharification of starch have been termed "limit dextrins."

Myrbäck (18) recognized the presence in barley malt of another enzyme besides the known alpha- and beta-amylases, this unidentified enzyme having the property of hydrolyzing limit dextrins to reducing sugars. Kerr and Schink (7) and Kerr, Meisel and Schink (8) demonstrated that an enzyme in fungal amylase preparations, designated by them "alpha-glucosidase," has a role in the conversion of certain limit dextrins. Kneen (10) reported the presence of such an enzyme, "dextrinase," in sorghum malt, and Kneen, Beckord and Kimura (11) reported on qualitative studies of this enzyme in barley malt, fungal and bacterial preparations. Corman and Langlykke (3) recognized the importance of a glucogenic enzyme in fungal preparations for the alcoholic fermentation of grain starches. Back, Stark and Scalf (1) and Kneen and Spoerl (12) developed quantitative methods for the estimation of this enzyme, now generally designated limit dextrinase.

In our laboratories over a period of years (23) special emphasis has been placed on cultivation of *Aspergillus oryzae* for production of enzyme products to be used in saccharifying starchy mashes for alcoholic fermentation by yeast. Since it is apparent that not only alpha-amylase, but also other enzymes produced by this mold, are factors in successful saccharification of starchy mashes for alcoholic fermentation, work was begun on the separation and isolation of carbohydrases from the culture material on which *A. oryzae* has been grown.

Active work in this general field has also been carried on in other laboratories. Using submerged cultures of *Aspergillus niger* NRRL-337, Lipps, Roy, Andreasen, Vernon and Kolachov (13) clearly demonstrated the presence of alpha-amylase, limit dextrinase, and maltase in filtrates from this mold. These workers, by fractional precipitation and adsorption procedures, separated the three components, obtaining a concentrate high in alpha-amylase activity but free of limit dextrin-

ase and maltase, one high in limit dextrinase activity free of alpha-amylase and maltase, and one with fair maltase activity free of alpha-amylase but still retaining considerable limit dextrinase activity.

It is the purpose of this paper to describe the methods which have been developed in our laboratories for the separation, purification, and crystallization of alpha-amylase and limit dextrinase from *Aspergillus oryzae*. Crystalline limit dextrinase has not previously been reported. Crystalline alpha-amylases have been obtained from pancreas (14, 15), saliva (16), bacteria (17), and malt (21). An enzyme of the alpha-amylase type has apparently not previously been isolated in crystalline form from a fungal source.

Materials and Methods

Through the courtesy of Enzymes Incorporated, Eagle Grove, Iowa, concentrated mold filtrate was made available as a source material for isolation of fungal enzymes. It was produced by submerged culturing of *Aspergillus oryzae* on a medium containing starch and inorganic salts, filtering the liquor and concentrating it by evaporation under reduced pressure. This material afforded a relatively concentrated source of mold enzymes in quantity lots and was therefore very convenient for this investigation.

Throughout the course of the work enzyme potencies were determined by the following methods: Alpha-amylase was determined by the method of Sandstedt, Kneen, and Blish (20), the activity being expressed as "units," representing the number of grams of starch dextrinized in one hour at 30°C. by the alpha-amylase in one milliliter of enzyme solution unless otherwise stated. The method of Back, Stark, and Scalf (1) was employed for determination of limit dextrinase, the activity being expressed as per cent hydrolysis of limit dextrin to fermentable sugar in one hour at 30°C. by 1 ml. of enzyme solution unless indicated otherwise.

Separation of Alpha-Amylase and Limit Dextrinase. Preliminary Purification. Examination of the concentrated mold filtrate disclosed that it possessed high activity of alpha-amylase and limit dextrinase, some maltase and some proteolytic activity. The absolute values of these various enzymes varied considerably from batch to batch of the concentrated mold filtrate as received. Since alpha-amylase is usually considered as the principal amylolytic enzyme produced by *A. oryzae*, efforts were initially concentrated upon the separation and possible crystallization of this enzyme.

The common method of separating enzymes from mold filtrates is precipitation by addition of alcohols (5). However, little if any separation of enzymes is secured by this precipitation. Caldwell,

Chester, Doebbeling, and Volz (2) prepared a maltase-free amylase from *A. oryzae* by repeated precipitation of aqueous extracts by use of ammonium sulfate, and found that other portein precipitants gave less satisfactory results. Ammonium sulfate precipitation was therefore adopted as the basis for our work. Various adsorption procedures and chemical treatments were also tried in combination with the ammonium sulfate precipitations in order to attempt separation of the enzyme components. After numerous trials resulting in many failures and some partial successes in separating the enzymes, finally a crystalline precipitate was obtained. Crystals of two distinctly different types appeared in this first crystalline precipitate. The procedure which resulted in the crystalline precipitate is outlined on page 22.

The precipitate labelled "C" in the above outline was free of maltase but showed both high alpha-amylase and limit dextrinase activity. All of the precipitates were examined microscopically as they were obtained, and none of them showed a crystalline nature until the one labelled "F" in the above flow diagram was examined. This precipitate was found to be crystalline and two very distinct types of crystals were apparent. Some of the crystals were of a hexagonal pyramid shape. Other crystals were long, shiny, silky needles. The crystalline precipitate was dissolved in a small amount of water, and the solution showed both high alpha-amylase activity and high limit dextrinase activity. In an effort to separate these components further precipitations were made as indicated in the flow diagram resulting in precipitates designated "H" and "I." Microscopic examination of the precipitate "H" showed only hexagonal pyramid type of crystals, and determinations on a solution of these crystals showed high alpha-amylase activity with much lower limit dextrinase activity than had been shown by any previous precipitates. Under the microscope the crystals of precipitate "I" were shiny, silky needles. Determinations on a solution of these crystals showed high limit dextrinase activity with relatively low alpha-amylase activity. It was therefore concluded that the hexagonal crystals were probably alpha-amylase and the needles were limit dextrinase, but both were still heavily contaminated with the other enzyme.

Efforts were therefore continued to completely separate the enzymes, and obtain them in crystalline form. After many trials success was finally achieved by the procedures given on page 24.

The initial steps in separating either alpha-amylase or limit dextrinase were the same. Starting with a large volume (50 liters) of concentrated mold filtrate three ammonium sulfate precipitations were made with a Bentonite adsorption interspersed after the first precipitation. This treatment eliminated extraneous protein material without

A large volume of concentrated mold filtrate chilled in refrigerator, solid ammonium sulfate added to three-fourths saturation. Kept overnight in refrigerator and decanted.

- Supernatant — (Discarded)
- A. Precipitate — Dissolved in one-tenth original volume of distilled water, solid ammonium sulfate added to three-fourths saturation. Chilled overnight and decanted.
  - Supernatant — (Discarded)
  - B. Precipitate — Dissolved in one-tenth former volume of distilled water, solid ammonium sulfate added to three-fourths saturation. Chilled overnight and centrifuged.
    - Supernatant — (Discarded)
    - C. Precipitate — Dissolved in minimum volume of distilled water and adjusted to pH 4.5 with 2 *N* acetic acid. Shaken with 5 g. of Bentonite per 100 ml. for two hours and centrifuged.
      - D. Supernatant — Solid ammonium sulfate added to three-fourths saturation. Chilled overnight and centrifuged.
        - Supernatant — (Discarded)
        - E. Precipitate — Dissolved in minimum volume of distilled water and two volumes of saturated ammonium sulfate solution added. Chilled overnight and decanted.
          - Supernatant — (Discarded)
          - F. Precipitate — (Two distinct types of crystals.) Dissolved in small amount of distilled water. Solid ammonium sulfate added to one-third saturation. Saturated solution of ammonium sulfate added gradually, with stirring, to slight turbidity. Kept at 30°C. for ten hours and centrifuged.
            - G. Supernatant — Solid sodium chloride added to three-fourths saturation. Kept at 30°C. for six hours and centrifuged.
              - Supernatant — (Discarded)
              - I. Precipitate — (Shiny, silky needles)
            - H. Precipitate — (Hexagonal pyramid crystals)
      - Solid — (Discarded)

excessive loss of enzymes. Representative data on the enzyme activities of the fractions in these preliminary precipitations are shown in Table I.

The original concentrated mold filtrate gave 9.5% hydrolysis of limit dextrin by the standard method, and had 18 units of alpha-amylase activity per ml. The first ammonium sulfate precipitation was carried out with five 10-liter portions of the concentrated mold filtrate. Each portion was three-fourths saturated with technical grade ammonium sulfate and kept overnight in the refrigerator. The precipitate from each was dissolved in 1 l. of water, and the solutions combined. As shown in Table I, one ml. of this solution gave 40.2% hydrolysis of limit dextrin and 179 units of alpha-amylase activity.

TABLE I

ENZYME ACTIVITIES OF FRACTIONS OBTAINED DURING INITIAL STEPS IN PURIFYING AMYLASE

Fraction	Limit Dextrinase, Hydrolysis	Alpha-Amylase	Alpha-Amylase, of Original
	%	*units per ml.*	%
A. Concentrated mold filtrate (50 l.)	9.5	18	100.0
B. First ammonium sulfate precipitate (in 5 l. soln.)	40.2	179	99.5
C. After Bentonite treatment (5 l. soln.)	38.6	168	93.3
D. Second ammonium sulfate precipitate (in 500 ml. soln.)	—	1,650	91.7
E. Third ammonium sulfate precipitate (in 50 ml. soln.)	—	16,100	89.4

This represents a recovery of 99.5% of the original alpha-amylase in the precipitate. Since the hydrolysis of limit dextrin was far above the range for proportionality demonstrated for their method by Back, Stark, and Scalf (1), calculation of the recovery of this enzyme cannot be made from the data.

The combined solution of the first precipitate was adjusted to pH 4.2 using 2 *N* acetic acid. Fifty grams of Bentonite were added to this solution and the mixture shaken for one hour, then centrifuged. The data of Table I show a slight loss of enzyme activity resulting from the Bentonite adsorption. The centrifugate from the Bentonite treatment was three-fourths saturated with solid ammonium sulfate, placed in the refrigerator overnight, and centrifuged. The precipitate was dissolved in 500 ml. of distilled water and the salting out procedure repeated. The precipitate from this was dissolved to make 50 ml. of

solution. Results of the alpha-amylase determinations after each precipitation are shown in Table I.

Crystalline Alpha-Amylase. Further steps in obtaining the crystalline alpha-amylase are shown in the following flow diagram, and Table II gives analytical data on the fractions obtained during this procedure.

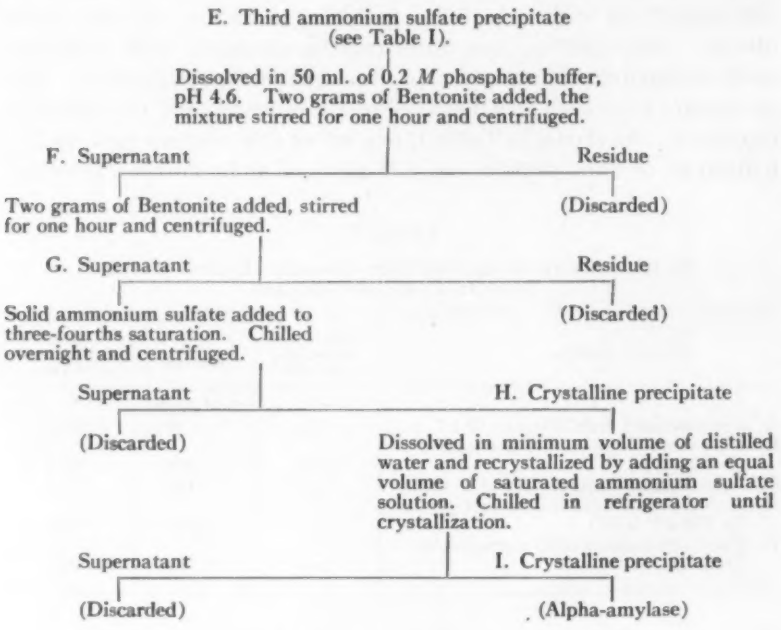


TABLE II

ENZYME ACTIVITIES OF FRACTIONS OBTAINED DURING STEPS IN CRYSTALLIZATION OF ALPHA-AMYLASE

Fraction	Limit Dextrinase, Hydrolysis	Alpha-Amylase	Alpha-Amylase, of Original
	%	*units*	%
E. Third ammonium sulfate precipitate (in 50 ml. soln.)	—	16,100	89.4
F. Solution after first Bentonite adsorption (50 ml.)	3.6	14,500	83.3
G. Solution after second Bentonite adsorption (50 ml.)	Trace	13,100	72.8
I. Final alpha-amylase crystals (1 mg. sample)	Trace	4,800	—

The final crystals which separated were of the characteristic hexagonal pyramid type. A typical photomicrograph of these crystals of alpha-amylase is shown in Fig. 1.

The data of Table II show that after the first Bentonite adsorption, analysis of 1 ml. samples of this solution showed 3.6% hydrolysis of limit dextrin and 14,500 units of alpha-amylase. After the second Bentonite adsorption only a trace of limit dextrinase activity remained and one ml. showed 13,100 units of alpha-amylase. After the final recrystallization a 0.01% solution (10 mg. of the crystals per 100 ml.) was prepared. One ml. of this solution gave only a trace of limit dex-

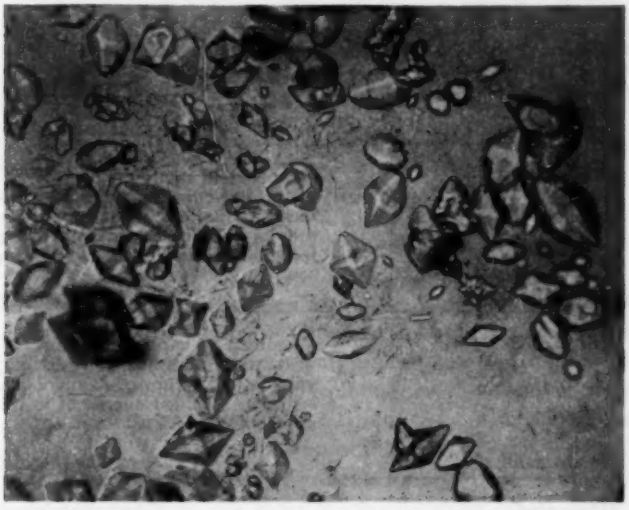
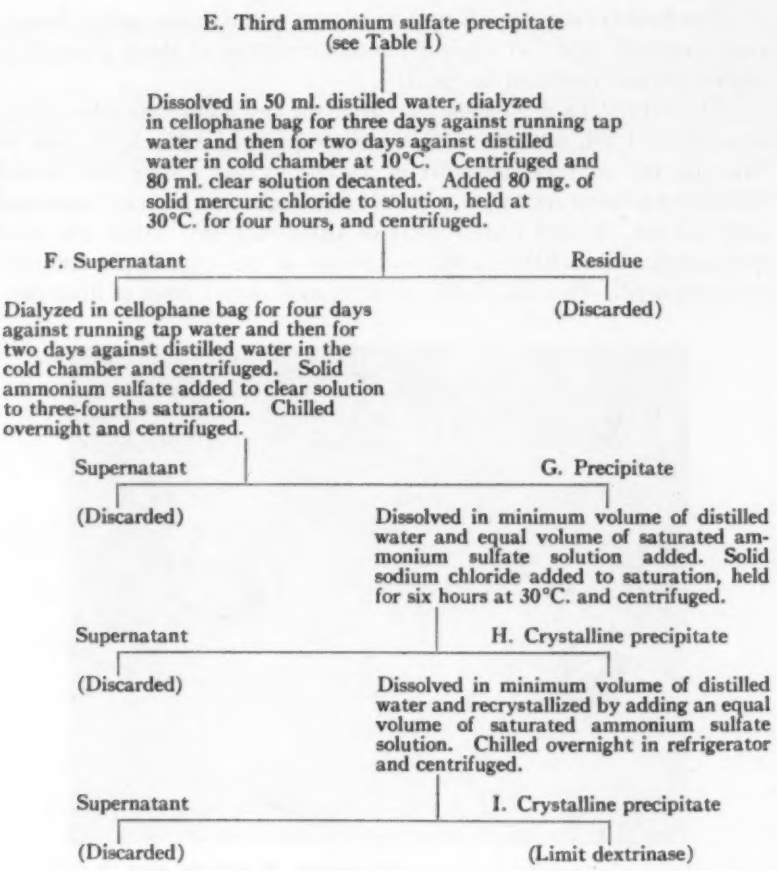


FIG. 1. Photomicrograph of alpha-amylase crystals. Magnification about 150X.

trinase activity, the hydrolysis by the standard method being less than 0.2%. A dilution of one ml. of the solution to 250 ml. was made, and one ml. of the dilution (0.0004 mg. of crystalline precipitate) had 1.92 units of alpha-amylase per ml. This represents an activity of 4,800 units per mg. of the crystals as shown in the table.

Crystalline Limit Dextrinase. For the preparation of crystalline limit dextrinase the initial steps were identical with those for alpha-amylase as described above and illustrated in Table I. The additional steps for the isolation of the crystalline enzyme are outlined in the following flow diagram, and analytical data on the various fractions are given in Table III.



The mercuric chloride treatment used in this procedure had been found in previous experiments to destroy alpha-amylase rapidly with only slight deleterious effect upon limit dextrinase under the conditions specified. After the treatment with mercuric chloride, dialysis was continued until practically no test for chloride was obtained. The crystalline precipitate designated "H" had the characteristic microscopic needle-shaped crystals. A photomicrograph of the final crystals obtained after recrystallization is shown in Fig. 2.

The analytical data given in Table III show that after the mercuric chloride treatment and dialysis the solution had a volume of about 100 ml. One milliliter of this solution was diluted to 100 ml., and 1 ml. of the dilution gave the analytical values shown in the table. The hydrolysis of limit dextrin was 48.7%, and alpha-amylase activity had

FIG. 2. Photomicrograph of limit dextrinase crystals. Magnification about 150×.

been reduced to only two units, corresponding to 2.2% of the alpha-amylase originally present in 50 l. of concentrated mold filtrate.

One ml. of a 0.01% solution (10 mg. of the crystals per 100 ml.) of the crystalline limit dextrinase produced 45.6% hydrolysis of limit dextrin, and had only a trace of alpha-amylase activity. When 1 ml. of the 0.01% solution was employed in the regular Sandstedt Kneen, and Blish procedure, after 60 minutes the test samples were still far from matching the end-point standard.

The losses during the extended process for crystallizing the enzymes were large, especially in the later steps involving small amounts of relatively concentrated solutions. Only a few milligrams of the crys-

TABLE III

ENZYME ACTIVITIES OF FRACTIONS OBTAINED DURING STEPS IN CRYSTALLIZATION OF LIMIT DEXTRINASE

Fraction	Limit Dextrinase, Hydrolysis	Alpha-Amylase	Alpha-Amylase, of Original
	%	*units*	%
E. Third ammonium sulfate precipitate (in 50 ml. soln.)	—	16,100	89.4
F. After mercuric chloride treatment and dialysis (100 ml. soln. diluted 1:100 for analysis)	48.7	2	2.2
I. Final limit dextrinase crystals (0.01% soln.)	45.6	Trace	—

tals of both alpha-amylase and limit dextrinase were obtained. The crystalline precipitates were dried *in vacuo*, and, of course, the dried crystals were contaminated with ammonium salts from the mother liquor from which they had been crystallized. Enzymatic activity of the crystals decreased slowly as they were kept in contact with the contaminating salts at room temperature. Deterioration was much slower at refrigerator temperatures.

Discussion

The preparation of crystalline alpha-amylase and crystalline limit dextrinase, particularly the latter, is of considerable significance. Availability of these enzymes in crystalline form should make possible their chemical characterization, and aid materially in studies on their modes of action. Work is now in progress on the preparation of larger amounts of the crystalline enzymes. It is hoped to secure better yields and to develop other final crystallizing procedures which will provide the crystalline enzymes free from contaminating salts. These larger quantities in purer form are desired for definite demonstration of specific activity upon recrystallization, electrophoretic studies and for chemical characterization of the crystalline compounds. The modes of action of the crystalline enzymes on various substrates will also be investigated.

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EXAMINATION OF STARCH DISPERSIONS WITH THE PHASE MICROSCOPE[1,2]

W. G. BECHTEL[3]

ABSTRACT

Corn starch was dispersed in alkali and by autoclaving, using methods given in the literature for the estimation of iodine affinity and for separation of starch fractions. Under the phase microscope the dispersed product was found to contain a large number of visible particles.

A suggested explanation is that the corn starch granule consists of carbohydrate chains of a wide range of molecular size and complexity. Some are held in dense, firmly bound regions, while others, less firmly bound, form the material between them. On gelatinization, those chains of low molecular weight and weak bond strengths dissolve. With more drastic treatment some of the larger and more complex starch chains are set free leaving the firmly bound regions relatively intact. The particles visible under phase microscope after the usual dispersive treatments probably form part of the amylopectin fraction.

In the literature on starch fractionation and the estimation of fractions by potentiometric titration with iodine, it is often stated or implied that the method of preparing the sample yields a complete dispersion or solution of the starch. For example, Schoch (15) has recently stated: "Briefly, the starch is pasted in hot water and the granule structure dissolved either by prolonged boiling or by auto-

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claving." Bates, French, and Rundle (1) stated: "Completely dispersed starch is a prime requisite in this method . . .", while Kerr (6) wrote ". . . complete solubility of the starch is reached at 125°C." It has sometimes been assumed that starch is completely dispersed when particles can no longer be seen under the ordinary microscope. Schoch (16) used viscosity as a test, continuing the dispersive process until a minimum viscosity was obtained.

With the ordinary microscope starch swollen in alkali or cooked in water becomes difficult to observe soon after swelling begins because the granules become transparent and lack sufficient contrast to bring out details of structure. This has been pointed out by Sjostrom (17), Woodruff (21), Schoch (14) and others. It has recently been shown (2) that the phase microscope gives a great increase in the contrast of swollen starch granules, permitting observations of structural details after long periods of cooking without staining with iodine and without reducing illumination to a point which makes observation difficult. For example, when unmodified corn starch was cooked at 95°C. for 4 hours at 1% concentration, the swollen granules and details of their structure appeared with a high degree of contrast under the phase microscope.

The present study was undertaken to employ the phase microscope for the examination of starches dispersed in alkali and by cooking in water. The samples observed were prepared by methods given in the literature for the estimation of starch fractions and for their separation.

Materials and Methods

Four samples of commercial unmodified corn starch were used. Two of these were defatted by the procedure of Schoch (13). By this means the fat content was reduced from about 0.9% to 0.24%. Another sample was defatted by a Soxhlet extraction for 27 hours using 95% ethanol. This treatment completely removed the fat. Fat analyses were made by the method of Taylor and Nelson (18). The fourth sample was used without pretreatment.

Dispersion in Alkali. For potentiometric titration with iodine, starch is first dispersed in potassium hydroxide. Several procedures are used in different laboratories. In order to study the dispersion of starch under conditions approximating those in general use the following methods were used: (1) Starch, 0.04 gm. in 10 ml. of 0.5 *N* potassium hydroxide at room temperature for 5 hours. (2) Starch, 0.1 gm. in 5 ml. of 1 *N* potassium hydroxide at room temperature for 24 hours. (3) Starch, 0.1 gm. in 5 ml. of 1 *N* potassium hydroxide at 0°C. for 6 days. Frequent microscopic observations were made during these periods.

Potentiometric Iodine Titrations. The method of Bates, French, and Rundle (1) as modified by Lansky, Kooi, and Schoch (10) was used.

Dispersion by Heat. The autoclaving method of Schoch (16) and that of Whistler and Hilbert (19) was used. In addition, one sample was cooked in a steam bath at 96°C. for 30 minutes at 1% concentration, then treated for 30 minutes in a Waring Blendor.

Microscopic Observations. A Bausch & Lomb phase microscope was used. Photomicrographs were made with the 8 mm. objective, 20× hyperplane ocular, and Bausch & Lomb type H microscope camera so adjusted that the magnification was 440×. Microscopic observations were made as rapidly as possible after the period of treatment to avoid possible effects due to changes on standing.

Results and Discussion

Dispersion in Alkali. As would be expected, the more drastic the treatment the more disintegrated the granules became. However, none of the alkali treatments yielded a dispersion free from innumerable microscopically visible particles, and there was no difference in this respect between the defatted starches and ordinary unmodified starch.

Initially the granules swelled rapidly, then more slowly to a diameter of 70 to 150 μ while the contrast diminished markedly. At the end of 1 or 2 hours the granules were still intact when 0.04 gm. of starch was treated with 10 ml. of 0.5 *N* potassium hydroxide at room temperature (Fig. 1). After 3 hours they were disintegrating (Fig. 2) and after 5 hours (Fig. 3) few whole granules remained. Although not readily apparent in Fig. 3, the liquid was filled with fragments of 5–10 μ in diameter. Stirring for various periods up to 1 hour at 200 r.p.m. with a propeller-type stirrer did not materially alter the fragments.

Similar observations were made when the starch was dispersed in 1 *N* potassium hydroxide at room temperature, except that the changes occurred more rapidly. Samples of starch given this treatment for 5 hours were then neutralized and warmed as suggested by Lansky, Kooi, and Schoch (10). At the end of 30 minutes warming the particles were still visible and the samples were boiled vigorously for 10 minutes, keeping the volume constant. Figure 4 shows the appearance of the product. The particles were not measured accurately, but from their size in the photomicrograph they appear to be from about 1 to 2 μ in diameter. At some places in the microscopic field streams of these particles were in motion. These had a fine, grainy appearance.

When starch in 1 *N* potassium hydroxide was dispersed at 0°C. the process proceeded at a much slower rate. In Fig. 5, made after a

4 hour treatment, the granules are seen to be largely whole though very wrinkled. With increasing time the granules gradually disintegrated until after 6 days they appeared as in Fig. 6, in which the visible particles are of irregular shape and vary from about 5 to 10 μ in dimension.

Potentiometric Iodine Titrations. To determine the effect of the state of swelling and disintegration of the granules on the estimation of fractions, potentiometric iodine titrations were made on samples dis-

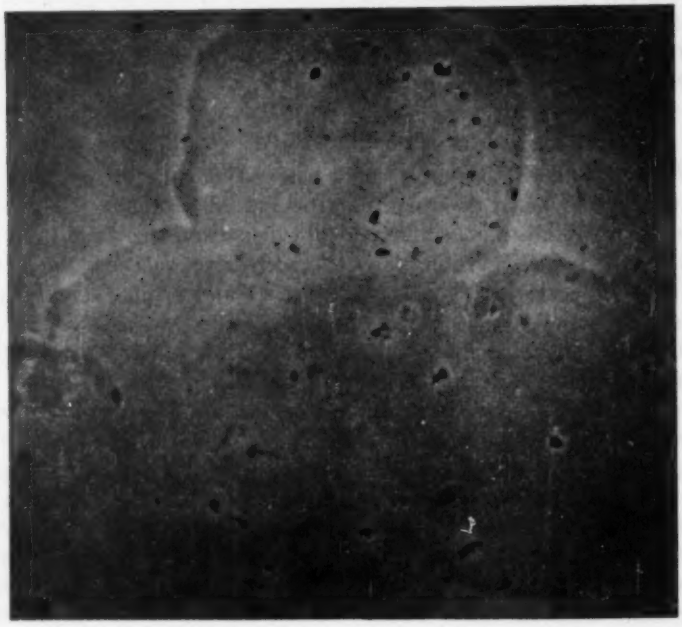

FIG. 1. Defatted corn starch treated with 0.5 *N* potassium hydroxide for 2 hours at room temperature. 440×.

persed in potassium hydroxide, 0.5 *N* for 2 hours, and 1 *N* for 1½, 4, and 24 hours. When bound iodine vs. free iodine was graphed, the points were found to lie along a single line in that part of the curve from which the iodine affinity was calculated, so that no significant differences in this quantity were found.

Apparently results of the potentiometric iodine titration do not depend on having a molecular dispersion of starch, but rather on the action of the alkali in loosening the bonds which hold the molecules together within the granule. It is also possible that in the greatly swollen granules which are present, the amylose fraction diffuses from the granules more rapidly than from those which are swollen in water.

This seems quite possible since the average diameter of granules when fully swollen in alkali was about one and one-half times that of the granules observed at 1% concentration in water after cooking at 95°C. until they were of maximum size.

Autoclaving. The method of Whistler and Hilbert (19) calls for cooked starch at 3% concentration in water to be autoclaved at 120°C. for 3 hours. Samples treated in this manner appeared as in Fig. 7. The

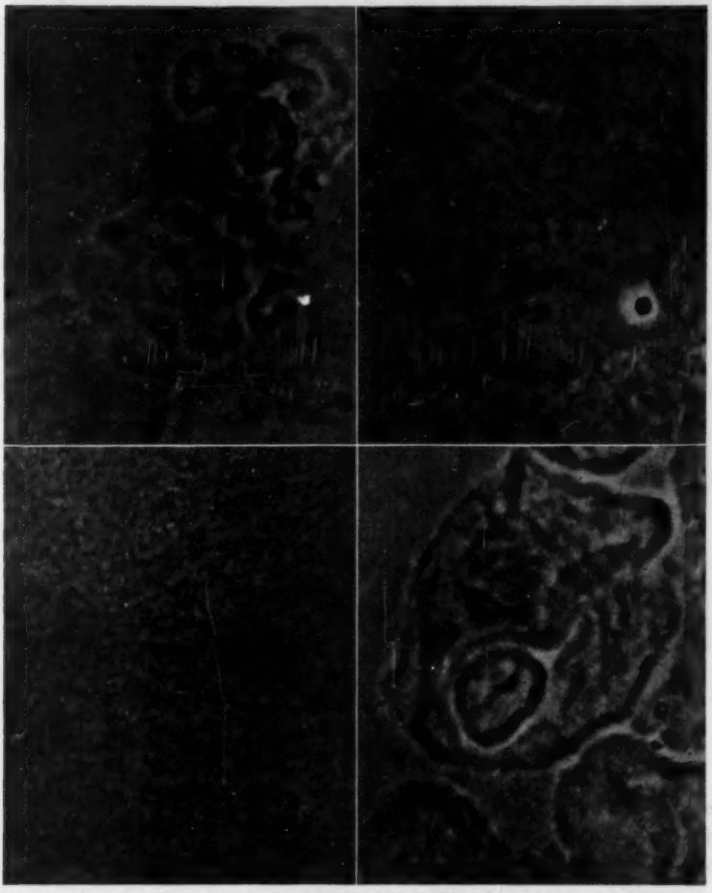

FIG. 2, top left. Defatted corn starch treated with 0.5 *N* potassium hydroxide for 3 hours at room temperature. 440×.

FIG. 3, top right. Defatted corn starch treated with 0.5 *N* potassium hydroxide for 5 hours at room temperature. The solution contained many fragments of 5–10 μ in dimension. 440×.

FIG. 4, lower left. Defatted corn starch treated with 1 *N* potassium hydroxide for 5 hours at room temperature, then neutralized and boiled for 10 minutes. 440×.

FIG. 5, lower right. Defatted corn starch treated with 1 *N* potassium hydroxide for 4 hours at 0°C. 440×.

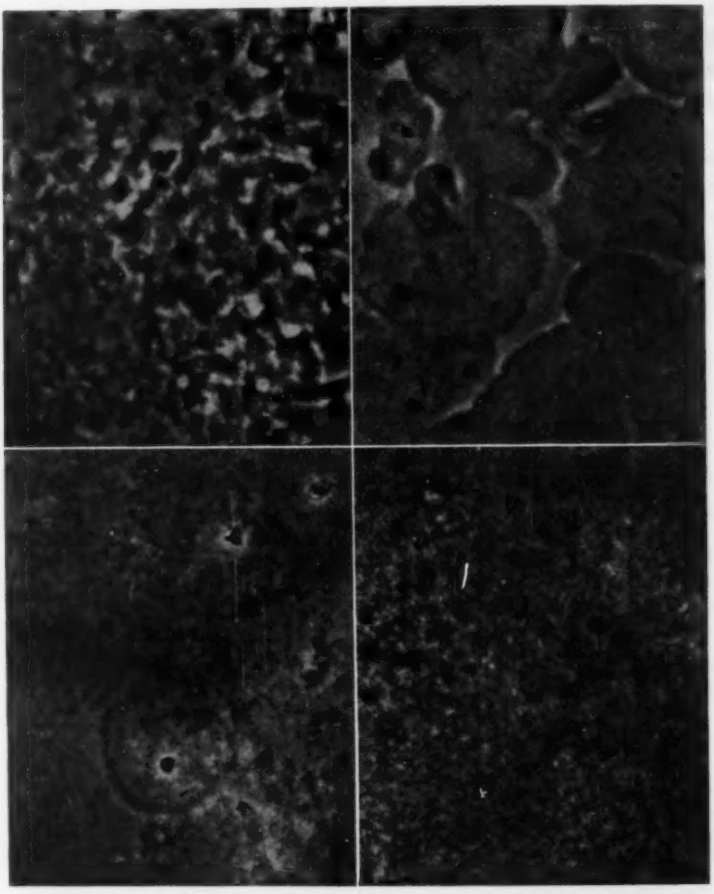

FIG. 6, top left. Defatted corn starch treated with 1 *N* potassium hydroxide for 6 days at 0°C. 440×.
FIG. 7, top right. Defatted corn starch autoclaved for 3 hours at 120°C. 440×.
FIG. 8, lower left. Defatted corn starch autoclaved at 124°–126°C. for 3 hours in the presence of butanol. 440×.
FIG. 9, lower right. Defatted corn starch autoclaved at 124°–126°C. in the presence of butanol. 440×.

product consists largely of swollen granules, though these must be disintegrated when they are passed through the supercentrifuge as recommended by the authors.

Schoch's method (16) differs from that above by employing a higher autoclaving temperature, 124°–126°C., and by use of butanol in excess of the amount required to saturate the water. The purpose of the butanol is to aid in the breakdown of granule structure and to prevent the formation of an insoluble "skin" on the surface. The effectiveness

of this process may be seen in Fig. 8, in which only vestigial traces of granules appear, although the liquid was filled with small visible particles. Figure 9 is another field in which the small particles which are present in vast numbers are brought sharply into focus. According to Wilson, Schoch, and Hudson (20) the starch so prepared may be treated directly with butanol and isoamyl alcohol to yield substantially the same fractions that are obtained when the product is passed through the supercentrifuge. On this basis it appears that the product which is fractionated consists, in considerable part, of particles of microscopic size.

A sample of this dispersion was centrifuged for 30 minutes at 2,000 r.p.m. The upper portion of the centrifugate then had the appearance shown in Fig. 10 in which the particles have evidently coalesced to form larger masses.

The photomicrographs used here are typical of results with both defatted and ordinary unmodified corn starch. In no case was an optically clear field obtained.

Cooking Followed by Treatment in the Waring Blendor. Starch samples were heated with water at 1% concentration in a steam bath until the starch was cooked at 96°C. for 30 minutes. The product was transferred to a Waring Blendor and treated for 30 minutes, after which it appeared as in Fig. 11, in which small round or oval particles are bound together by a surrounding visible medium. The number of particles which are visible is, however, much smaller than in auto-

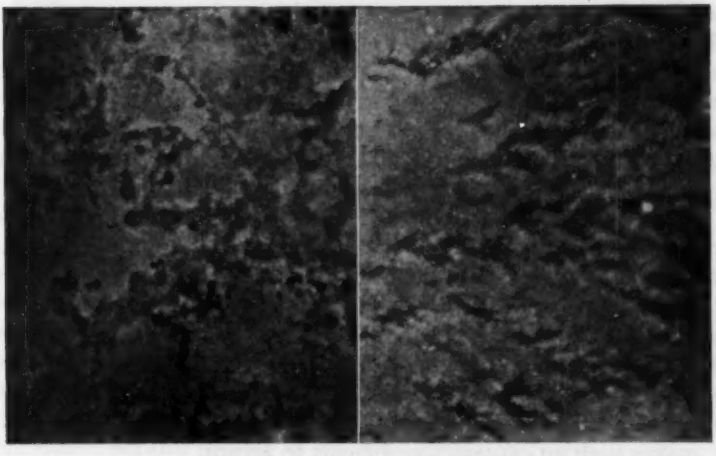

FIG. 10, left. Defatted corn starch autoclaved at 124°–126°C. in the presence of butanol, then centrifuged for 30 minutes at 2,000 r.p.m. 440×.

FIG. 11, right. Defatted corn starch cooked for 30 minutes at 96°C., then treated for 30 minutes in a Waring Blendor. 440×.

claved and alkali-treated starches which are not subjected to violent shearing action.

Treatment with Amylase. Starches dispersed by the above methods and with pH suitably adjusted, were treated with the commercial amylase Vanzyme. At intervals microscopic examinations were made and simultaneously samples were treated with iodine. As the color with iodine diminished in intensity the visible matter became less. It disappeared, leaving a void field, at about the same time that the color with iodine disappeared.

Granule Structure. An orderly arrangement of particles within slightly swollen granules has been observed by a number of investigators including Samec (12), Sjostrom (17), Woodruff and MacMasters (22), who gave a review of the literature on this subject, and Hanson and Katz (4). Hanson and Katz described an orderly concentric and radial arrangement of globular units of about 1 μ in diameter in the granule with an intermediate substance between them.

From the present study it is concluded that such particles are constituent parts of the corn starch granule and that they are set free in large numbers when starch is given sufficiently drastic treatment in aqueous alkali or by cooking in water. In Fig. 12 a granule of undefatted starch appears in the process of disintegration after being cooked for 2 hours at 91°C. in a 1% suspension in water. It shows a grainy texture with a large number of small, optically-dense particles joined by a less dense material. These patricles are visible in the granule and may be seen streaming out of it. Figure 13 shows the granules of a defatted corn starch sample that had been treated with 0.5 *N* potassium hydroxide for 4½ hours. At the end of that period the swollen granules showed no evidence of disintegration and the sample was given a gentle shaking. This was sufficient to start the disintegrating process. The internal structure is shown with a large number of particles bound in place throughout the granules. Some have been set free and are in the surrounding fluid.

Such particles appear more or less clearly in all of the photomicrographs of sufficiently dispersed starch. They are readily seen in Fig. 4, after the most drastic treatment with alkali, in Figs. 8, 9, and 10 after autoclaving, in Fig. 11 after treatment in the Waring Blendor, and in Figs. 12 and 13. If they appeared only as an end-product of autoclaving in the presence of butanol it might possibly be concluded that they were microcrystals of amylose-butanol complex. However, their appearance after drastic treatment with alkali as shown in Figs. 4 and 13, makes such an explanation difficult to accept.

Starch Fractions. It is evident that the methods which have been used for fractionating starch do not disperse corn starch granules com-

pletely into molecules. Therefore, such methods cannot yield the two pure fractions amylose and amylopectin (or A-fraction and B-fraction). Higginbotham and Morrison (5) recently reached the same point of view as the result of attempts to fractionate corn starch and purify the fractions. By use of a colorimetric method for determining the proportion of amylose present they concluded that only about 76% of the amylose of corn starch was precipitated by butanol from autoclaved pastes and that when dispersion of the starch *appeared* to be complete, aggregates were present which included about 20% of the amylose and 80% of the amylopectin. They suggested that the aggregates would become part of the amylopectin fraction, or would be removed from crude amylose on its recrystallization. The stability of the aggregates to drastic treatment led them to believe that ordinary valence bonds were involved rather than van der Waals' forces or hydrogen bonds.

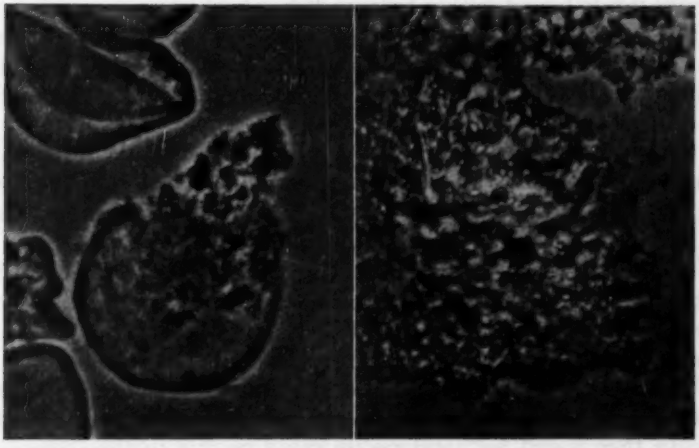

FIG. 12, left. A corn starch granule (not defatted) disintegrating after being cooked for 2 hours at 91°C. 440×.

FIG. 13, right. A granule of defatted corn starch disintegrating after treatment for 4½ hours with 0.5 *N* potassium hydroxide followed by gentle shaking. 440×.

Kerr and Severson found (9) that if corn starch was extracted with water several times at the same temperature, the yield of soluble starch did not follow a solubility equilibrium. Instead there was a definite proportion of the starch which dissolved readily at a given temperature, regardless of the amount of water used. Kerr (8) also found that if starch was extracted with water at successively higher temperatures more starch became soluble as the temperature increased.

Recently Lansky, Kooi, and Schoch (10) reported that the iodine affinity of amylose is not constant, but varies with the natural variety

of the starch, the batch prepared, and the method of fractionation and crystallization. Amylopectin from corn starch was found to have values of iodine affinity varying from 0.3 to 0.9%. They also found that the yield of Pentasol-precipitated corn amylose was 28–29% while that of the butanol-precipitated product was only 22–23%. These and other irregularities they attributed to a possible new starch fraction intermediate between amylose and amylopectin, which would comprise about 5–7% of the total starch substance.

In view of the present evidence it seems reasonable to conclude that corn starch granules consist of carbohydrate chains of a wide range of molecular weights and complexity of structure, some of which are held together in dense, firmly bound regions, while others, less firmly bound, form the material between them. When starch is heated with water to or above the gelatinization temperature the bonds of lowest energy are broken, the granules swell, and a certain proportion of the chains which are less firmly bound (either because of bonds of lower energy or fewer bonds) break completely away and dissolve. Van der Waals' forces, hydrogen bonds (3), and the acetal and hemiacetal bonds suggested by Pacsu (11) may all be involved, in addition to primary valence bonds of high energy.

This would be in accord with the findings of Kerr and Severson (9) that there is a definite limit to the amount of starch which dissolves at a given temperature regardless of the amount of water used. Those carbohydrate chains of lowest molecular weight and bound in the granules by the weakest bonds would be the first to be set free. It does not seem likely that the partial solution of starch under these conditions is accomplished, as suggested by Kerr (7), simply by mechanical disentanglement of already separate molecules.

The irregularities in properties of starch fractions found by Lansky, Kooi, and Schoch can likewise be explained on this basis. The iodine affinity they found for amylopectin could be due to the presence in this fraction of that part of the starch which remains of microscopic size after autoclaving, or which has not been broken into fragments of small enough size to precipitate as a complex with higher alcohols or other complexing agents. The increased yield of amylose when precipitated with Pentasol, and its lower iodine affinity compared to that obtained with butanol could be due to the Pentasol-amylose complex being less hydrophilic than the butanol-amylose complex and thus precipitating an additional quantity of starch molecules of greater molecular size and complexity. This would also explain why they found that recrystallization of amylose with Pentasol yielded a product of lower iodine affinity than when butanol was used for recrystallization.

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THE THICKNESS OF WHEAT BRAN [1]

JOAN CREWE AND C. R. JONES [2]

ABSTRACT

The mean thickness of bran at ordinary moisture contents (13–18%) was found to be 67 μ, regardless of the type of wheat, that of the aleurone layer being 30–36 μ. Through swelling, particularly of the outer layers, the mean total bran thickness in wet-mounted sections is in the range 100–109 μ; that of the aleurone layer is about 46 μ. Considerable variation occurs between individual grains but average values are similar for various types of wheat. Size of non-shrivelled grain is only slightly connected with thickness of outer bran layers and not at all with that of the aleurone layer, but immature, shrivelled grains of Manitoba wheat have thicker bran than mature grains.

The swelling of bran becomes marked only at moisture contents above 30%. It is similar in water, in salt solutions and in 5% glucose solution.

Within individual grains the aleurone layer cells may show marked irregularity in thickness (in transverse sections), often forming steps between adjacent cells. The extent and nature of the irregularity varies with different types of wheat and with the position in individual grains. It is a factor causing apparent variation in bran thickness and is one of the factors determining ease of detachment of endosperm from bran during milling.

Although botanically, the aleurone layer forms part of the endosperm, it normally remains attached to the outer layers of the grain during milling and (apart from any adherent endosperm) is the innermost layer included in the bran. The thickness of wheat bran, including the aleurone layer, is therefore important in affecting flour yield (for discussion see Scott (6)). Available data for thickness are summarized in Table I.

They appear mainly (apart from those of Shellenberger and Morgenson) to have been obtained on single grains of different types of wheat and are scanty and conflicting; this is particularly confusing in that the values (63–66 μ) for air-dry bran differ markedly from those (100–180 μ) from microscopical measurements of mounted sections. The difference is obviously due to swelling of the tissues in the aqueous preparations.

The values of Shellenberger and Morgenson (7) are nearer to those for air-dry bran than to the other microscopically determined values, evidently because these workers dehydrated their sections of initially

[1] Manuscript received May 18, 1950.
[2] The Research Association of British Flour Millers, St. Albans, England.

moistened grain. Shellenberger and Morgensen confirmed Bates's conclusion that certain types of hard red winter wheat have thicker brans than others, though the differences found were relatively slight and were not related to flour yield. Their results also indicate slight differences in bran thickness between wheats of similar type grown at different localities and also between those grown in different seasons.

On the other hand the non-visual measurements of Scott, and of Bayles and Jones, on air-dry bran showed that very different types of wheat (e.g., Manitoba and soft English) have similar bran thicknesses.

TABLE I

SUMMARY OF WHEAT BRAN THICKNESS DATA IN THE LITERATURE

Workers	Literature Reference	Thickness	
		Aleurone Layer	Whole Bran
		Aleurone Layer and Whole Bran, Microscopically Determined	
		μ	μ
Percival	4	65–70	Say 122–135[1]
Fairclough	3	44–53[2]	110–180
Bates	1	22–37[2]	97–143
Shellenberger and Morgenson	7		64–78
		Air-Dry Bran, Directly (Non-Visually) Measured	
Willard and Swanson	8	—	63.5[3]
Scott	6	—	66 [4]
Bayles and Jones	2	—	64 [5]

[1] Obtained by adding a reasonable value of 12–15 μ for thickness of layers between the pericarp and aleurone layer to Percival's figures for the aleurone layer and for the pericarp (45–50 μ).
[2] Obtained by measurement from the drawings or photomicrographs given.
[3] Recorded as "$\frac{1}{400}$ in."
[4] By micrometer measurements.
[5] By dial gauge on pieces of bran complete with aleurone layer but freed from adherent endosperm by manipulation under the microscope.

Attention does not seem to have been called to the fact that the values based on microscopy of mounted sections may have no relation to grain conditioned for milling. Moreover, assuming that all air-dry grains have equally thick bran (about 65 μ), the variability in the data for wet mounts implies that the tissues of different wheats swell to different extents. Experimental work was therefore undertaken in which the thicknesses of the aleurone layer and of the total bran were measured by the same means (microscopically) both in dry and in wet sections.

Materials and Methods

Grains of No. 1 and No. 2 N. Manitoba, Australian, Baril Plate, Yeoman English, soft red and soft white English, and of English-grown Rivet and Bersée wheats were examined.

Moisture contents, which are expressed on the wet or sample basis, were determined by ascertaining the loss in weight on drying the coarsely ground wheat for 4 hours at 120°C. In the case of wet samples a preliminary air-drying of the initially weighed specimen was made.

In the case of "dry" sections, determinations of thickness were made to the nearest micron in two ways:

(a). The air-dry grain was simply bisected transversely and examined under an Ultropak vertical illuminator with the aid of an eye-piece micrometer.

(b). Grains at approximately 18% moisture content were wax-embedded and sectioned transversely midway along the grain by means of a sharp razor. It was not possible to cut reasonably thin sections without this amount of moistening. The sections which were 20–25 μ in thickness were mounted in clove oil and viewed ordinarily, with transmitted light, again using the eye-piece micrometer.

In the case of wet-mounted sections, the procedure was as in (b) above, except that the specimen was mounted in water instead of in clove oil.

With the dry sections, and in the preliminary work on wet sections, varying numbers of representative grains (in some cases a dozen) were examined. Usually four measurements were made, on a single section from each grain, by each of the three methods mentioned, but several sections from some individual grains were examined. The measurements were spaced as regularly as possible around the exposed surface of the grain (measurements were not made on the bran in the crease).

Results and Discussion

Dry Sections. With the dry sections of all types of wheat, methods (a) and (b) gave generally similar results, showing no appreciable swelling to be caused by raising the moisture content to 18% from the air-dry state of 13–14%. For all types of wheat, measurements of the total bran thickness lay within the range 50–77 μ, with a mean of 67 μ (which is close to the figures, from non-visual determinations, given in Table I) and no appreciable difference between different varieties of wheat was found. The thickness of the aleurone layer was mostly within the range 30–36 μ (average 33) though occasional departures from this close range occurred, the extreme values found being 24–40 μ.

TABLE II

WEIGHTS OF INDIVIDUAL GRAINS OF DIFFERENT WHEAT TYPES

Wheat Type	Large Grains		Small Grains	
	Range	Mean	Range	Mean
	mg.	*mg.*	*mg.*	*mg.*
No. 2 Manitoba	40.0–45.8	43.6	21.8–27.9	24.7
Yeoman	—	54.4	—	39.1
Bersée	64.6–77.2	68.8	41.9–50.0	45.3

Wet Sections. A preliminary series of measurements showed that, with water-mounted sections, considerable variation in bran thickness occurs between individual grains and within individual grains; in comparison with this variation, no significant differences were found between different types of wheat. Further the thickness was not markedly affected by variation in size of grain.

To test these conclusions by means of statistical analysis, a series of similar sets of measurements was made on three dissimilar types of grain: No. 2 Manitoba, Yeoman English and English grown Bersée. The Bersée was included because it is considered by practical men to

TABLE III

THICKNESS OF ALEURONE LAYER AND OF WHOLE BRAN LESS ALEURONE LAYER IN WATER-MOUNTED SECTIONS OF INDIVIDUAL GRAINS OF MANITOBA WHEAT

Grain No.	Aleurone Layer					Whole Bran less Aleurone Layer				
	Measurements				Mean	Measurements				Mean
	a	b	c	d		a	b	c	d	
Large Grains										
	μ	μ	μ	μ	μ	μ	μ	μ	μ	μ
1	46	45	46	49	46.5	49	47	69	66	57.8
2	42	44	46	50	45.5	53	54	50	66	55.8
3	53	53	46	45	49.2	59	53	53	58	55.8
4	48	46	48	41	45.8	64	73	64	66	66.8
Means					46.8					59.0
Small Grains										
1	46	53	53	48	50.0	53	50	58	55	54.0
2	42	48	54	50	48.5	64	58	54	61	59.3
4	42	42	38	44	41.5	57	53	58	52	55.0
4	55	55	45	49	51.0	51	39	51	50	47.8
Means					47.6					54.0

be exceptionally thick-skinned. Within each wheat type four large and four small, mature (not shrivelled) grains were examined, and on each grain four representative measurements of the thicknesses of the aleurone layer and of the whole bran were made. The measurements were representative, rather than random, in the sense that exceptional cells, obviously much larger or smaller than the great majority, were avoided. The grains were weighed individually. The ranges and mean weights in mg. for the large and small grains of each type are shown in Table II.

Table III shows, by way of example, the data for thicknesses of aleurone layer and of whole-bran-less-aleurone layer on the individual Manitoba grains, and Table IV shows the results of statistical treatment of the data for all three types. The following conclusions may be drawn from Table IV.

TABLE IV

ANALYSIS OF VARIANCE OF THE THICKNESS OF ALEURONE LAYER AND WHOLE BRAN LESS ALUERONE IN MANITOBA, YOEMAN, AND BERSÉE WHEATS

Source of Variance	Degrees of Freedom	Variance	
		Aleurone	Bran less Aleurone
Between wheat types	2	75	66.5
Between sizes	1	12	460[1]
Types × sizes	2	30	19
Between grains	18	36[2]	81.5[2]
Within grains	72	15	29.6
Total	95		

[1] Denotes 5% level of significance.
[2] Denotes 1% level of significance.

Variation in thickness of aleurone layer between grains (within a wheat type and a size class) is significantly greater than variation within grains. The thickness of the aleurone layer of the large grains is not significantly different from that of the small grains. Nor are the differences between wheat types significantly greater than the differences between grains.

Variation in thickness of the whole bran less the aleurone layer is also significantly greater between grains than within grains. Although the thickness of the aleurone layer did not appear to be associated with grain size, the data for bran do indicate a slight connection, particularly with Manitoba and Yeoman. The mean values for whole bran less aleurone layer in large and in small grains of the three types were:

	Manitoba	*Yeoman*	*Bersée*
	μ	μ	μ
Large	59.0	60.5	60.5
Small	54.0	55.5	58.0

TABLE V

MEAN THICKNESS OF ALEURONE, BRAN LESS ALEURONE AND WHOLE BRAN IN WATER-MOUNTED SECTIONS OF WHEAT GRAINS OF ALL TYPES

	Mean Thickness		
	Aleurone	Bran less Aleurone	Whole Bran
	μ	μ	μ
Large grains	46.6	60	106.5
Small grains	46	55.5	101.5
All grains (mean value)	46	58	104
Range of values	32–55	39–73	—
Standard deviation	4.6	7.0	—
S.E. of mean	± 1.04	± 2.18	—

Differences in bran thickness between wheat types are again not significantly greater than the differences between grains.

The average values (in microns) representing about one hundred measurements of thickness on all three types of wheat, are shown in Table V.

These data may appear surprising in two respects:

1. That all types of wheat examined have brans of similar thickness. This finding opposes a strong practical belief, and it must be admitted that visual and tactual experience in milling suggests that some wheats are thicker-skinned than others. The explanation may be two-fold:

(a). Wheats considered thick-skinned are those whose endosperm can be less readily separated from the bran, which usually carries adherent endosperm when handled.

(b). In some wheats, particularly Bersée (reputed to be exceptionally thick-skinned), the epidermis tends to separate partially from the inner layers of the bran, causing air spaces to be included within the apparent thickness of the bran. Superficially such bran *is* thicker, to an irregular extent, than those where no separation occurs, but of course it is not so in respect of total thickness of actual tissue, which is the determining factor in regard to the proportion by weight of endosperm in the grain.

2. That the difference in bran thickness between large and small grains of any one type is so slight. It must however be remembered that no significant difference was found between wheat types, where the difference in size of grain may be considerable, e.g., Manitoba 43.6 mg., Bersée 68.8 mg. With grains of any one type the difference is much smaller proportionally than the difference in size of the grains. Thus the (dry) large Manitoba grains had a mean radius of 1.7 mm., and the small, 1.4 mm. The difference (0.3 mm.), expressed as a percentage

of the larger value, is 17.5. A proportional difference in total bran thickness would thus be 18.2 μ, as against the observed difference (*vide* Table III) of 4 μ. It follows that larger grains must contain a higher proportion by weight of endosperm than smaller grains of similar shape.

On the other hand immature grains, in the form of small *shrivelled* grains found in No. 2 Manitoba wheat, were found to have greater bran thickness than mature grains. Thus five such shrivelled grains, of mean weight 18.8 mg. (range 18.0–21.0), showed a mean thickness of 57 μ (range 50–70.5) and a mean total bran thickness of 129 (range 110–150). These thicknesses compare with 47 and 105 μ, respectively, for normal Manitoba grains.

The Swelling of Bran. From the data given the following figures may be calculated for swelling of aleurone and bran less aleurone, expressed as percentages of the dry thicknesses.

	Thickness in μ		
	Dry	*Wet*	*Swelling (%)*
Aleurone	33	46	40
Bran less aleurone	34	58	70
Whole Bran	67	104	55

The exterior layers thus swell proportionally more than the aleurone.

There is no indication of a significant difference in the extent of swelling with different types of wheat.

Further experiments, which need not be reported fully, were made on transversely bisected grains adjusted to various moisture contents by exposure to water vapor of different degrees of saturation. They showed that:

1. The onset of marked swelling of bran occurs only at moisture contents greater than 30%, i.e., well above the range of ordinary practical interest. The water content of the specimens mounted in water was about 50%.
2. Transverse swelling of the material in thin sections is similar to that shown in bisected grains, suggesting that the swelling is not constrained by the bulk of the grain.

Solutions of glucose and of salt have sometimes been used as mountants to prevent distortion in sections of grain. It was ascertained however that the extent of bran swelling in 5% glucose, and in 5, 10 and 25% sodium chloride solutions, is similar to that in water.

Variation or Irregularity in Aleurone Layer. Considerable variation in the thickness of the aleurone layer occurs which naturally affects the total thickness of the bran. In fact the aleurone cells are never completely uniform in thickness in any type of wheat.

As a general rule, in transverse grain sections, whilst the aleurone layer consists of reasonably uniform cells along the faces of the two cheeks, variation tends to occur at the sides and towards the dorsal face of the grain. This variation is more noticable in some types of wheat than others. Where considerable variation occurs it can be sufficient to account for the variation shown in total bran thickness. The total bran thickness may however vary where the aleurone layer is uniform, i.e., the thickness of the pericarp can also vary slightly.

In some wheat types a definite irregularity in the size of adjacent cells can be seen giving a step-like or even crenellated appearance. This appearance, which does not seem previously to have been pointed out, is shown in Fig. 1.

TABLE VI

MEAN "STEP," IN MICRONS, BETWEEN ADJACENT ALEURONE CELLS

Wheat Type	*Step*	
Soft Red English	12.0	
Karachi (white grains)	10.9	
Yeoman	8.8	Mean 7.74±1.26
W. Australian	7.9	
Baril Plate	7.8	
Rivet	4.1	
Manitoba	3.5	
Source of Variance	*Degrees of Freedom*	*Variance*
Between varieties	6	115.3
Within varieties	68	29.6
Total	74	

Series of eight measurements of thicknesses of adjacent aleurone cells were made on two representative grains of each of several wheat types. Table VI shows the mean "steps" in microns between adjacent cells for each type. Although relatively few measurements were made, the results of the statistical analysis confirmed impressions from a preliminary survey that wheat types differ in the regularity of their aleurone cells. Those of the soft red English, Karachi and Yeoman wheats are significantly more irregular than those of the Rivet and Manitoba. Although English-grown, and of poor baking quality, Rivet (*T. turgidum*) is characteristically easy to mill, the endosperm being "lively" and granular and the bran readily cleaned. It is of interest that the wheat types happen to fall into much the same order in respect both of milling quality and of uniformity of aleurone cell thickness. The brans which are easiest to free from adherent endosperm during milling have the smoothest aleurone-endosperm profiles and vice versa.

It is probable that, to some extent, the degree of smoothness of the profile affects directly the ease of separation of endosperm from bran by the last break rolls. Thus, transverse sections were made of (wax-embedded) pieces of bran delivered from the last (fifth) break rolls of a laboratory milling system adjusted to extract 70% of flour from mixed English wheat. The appearance of the adherent endosperm was, typically, as drawn in Fig. 1. Endosperm tended to remain attached to larger, projecting cells as at (a) in Fig. 1, whereas groups of smaller uniform cells were almost completely free of endosperm.

Where one or two small cells are "sandwiched" between larger cells, a small portion of endosperm is almost always to be found attached, as at (b) in Fig. 1. Also where adjacent cells occur in steps endosperm is often to be found in small quantities.

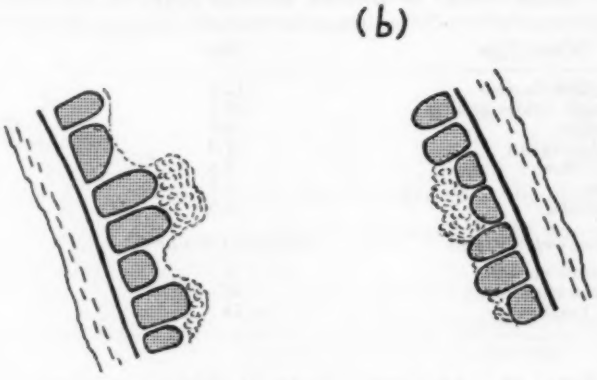

FIG. 1. Milled wheat bran in transverse section.

It is not claimed that the factor is a major one. While some pieces of bran carry little adherent endosperm, others, delivered from the same milling system, may be coated with an endosperm layer of thickness equal to, or greater than, that of the accompanying bran. Such gross differences are doubtless dependent on other properties of the wheat grain. It is merely recorded here that with bran pieces which have been largely freed from endosperm by the break rolls, the residual endosperm tends to be in aggregates whose position is associated with irregularities in shape of the aleurone cells.

The phenomenon recalls Sandstedt's study (5) of Gordon's theory that the aleurone layer is a meristematic tissue whose function is the production of endosperm cells. The fact that we find that endosperm tends, rather unexpectedly, to adhere to large projecting aleurone cells may be due to some particular phase in the connection between the

latter and the outermost endosperm cells which have just developed from them. It is tempting, too, to suggest that the more uniform aleurone layer of the harder wheats, whose bran cleans more easily, may be due to cessation of formation of new endosperm cells earlier in the development of the grain.

Acknowledgment

The authors are indebted to Dr. N. L. Kent for the statistical analyses.

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AMYLASE OF BACILLUS MACERANS [1,2]

Walter S. Hale and L. C. Rawlins

ABSTRACT

A scheme for growing *Bacillus macerans* on a laboratory scale larger than usual has been devised, and with its help an 8-fold purification (with respect to protein) of the macerans amylase in filtered culture fluid has been accomplished. The most effective step in the purification has been adsorption of the enzyme on raw starch.

The properties of the purified material indicate that a single enzyme is effective in producing Schardinger dextrins not only from starch but from the intermediate products of ordinary amylolysis. Its rate of action on starch is decreased by β-Schardinger dextrin and accelerated by glucose or maltose.

The crystalline dextrins first found by Schardinger (11, 12) when *Aerobacillus macerans* was grown on starchy media have been shown by Freudenberg and Meyer-Delius (5) to consist of closed rings of glucose units in 1,4-α-glycosidic linkage. Such a ring structure is not regarded as inherent in the starch molecule, which possesses essentially an open

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[2] Enzyme Research Division Contribution No. 128 Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture, Albany, California.

chain structure. However, Tilden and Hudson (15) and McClenahan, Tilden and Hudson (10) have demonstrated that the Schardinger dextrins are not excretory products of the organism, but are formed from starch through the action of an enzyme soluble in the culture fluid. Accordingly, the formation of Schardinger dextrins must have a bearing on the constitution of starch itself. Furthermore, as the Schardinger dextrins possess no reducing action, and are not fermentable by yeast (11), their occurrence may well explain obscure losses of starch in a variety of industrial processes, particularly since the organism responsible is of common occurrence in air, soil, and water.

Schardinger isolated two distinct crystalline dextrins (α and β) from cultures of macerans grown on potatoes. These make up the greater part of the usual product. Gamma dextrin (isolated by Freudenberg and Jacobi (6)) has also been obtained recently by French, Levine, Pazur, and Norberg (2, 4), and French and Rundle (3). Minor amounts of still other dextrins have also been found. Thus Freudenberg and co-workers (7) have described the separation of five distinct substances. The α-dextrin contains six glucose units, and the β-dextrin seven.

Macerans amylase, the enzyme held responsible for the formation of Schardinger's dextrins, has been considerably concentrated and purified by Tilden and Hudson (15). Further purification seems desirable, however, before the reaction can be studied in its simplest form. The presently reported work has led to methods for growing the organisms on a relatively large scale in submerged cultures, to a more convenient method of assay for the enzyme, to its further purification, and to some observations on the purified material.

Materials and Methods

Measurement of Enzyme Activity. A method for the assay of macerans amylase was described by Tilden and Hudson (15) based on the time required for the enzyme preparation to form enough dextrin (from starch) to produce characteristic crystals of a complex of dextrin and iodine. At this point the color of the digest after the addition of iodine is no longer blue but brown-violet. The following method is a modification of the foregoing, in which the enzyme quantity is calculated from the time required to reduce the opacity of the digest, when treated with iodine, to a transmissibility of 50%. The procedure is the same as that used by Schwimmer (13) for α-amylase. The method does not differentiate the action of macerans amylase and that enzyme. However, the macerans preparations used in this work did not appear to contain any appreciable amount of α-amylase, as judged (1) by the non-appearance of reducing substances, and (2) by the

approximate parallelism throughout a considerable range of purity between the results of this method and the crystal method of Tilden and Hudson (which does differentiate between macerans and α-amylases).

In detail, the assay is made as follows:

One ml. of *M*/1 calcium acetate-acetic acid buffer, pH 5.2, and 4.0 ml. of water containing the enzyme are added to 10 ml. of 2% starch (Lintner soluble) solution at 40°. After 5, 10, or 20 minutes (depending upon the activity of the enzyme), 0.5 ml. of the digestion mixture is removed, and added to 5 ml. of a solution of iodine in potassium iodide (.0035 *M* I_2 in 0.25 *M* KI). This mixture is then diluted by the addition of 10 ml. of H_2O, and the light transmission of the diluted

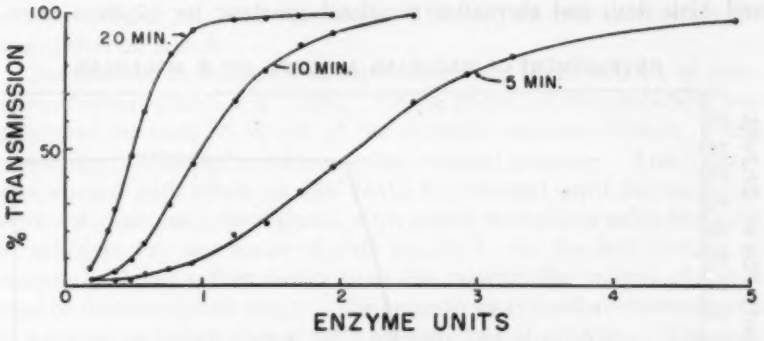

FIG. 1. Units of macerans amylase based on 50% transmission of light at 660 mμ through the edxtrin-iodine solution after 5, 10, and 20 minutes incubation, using increasing concentrations of the nezyme with 2% soluble starch as described in the text.

mixture is measured at 660 mμ in an Evelyn photoelectric colorimeter at 25° three minutes after the addition of the iodine. The colorimeter scale is set so that the blank containing iodine, but no digest, reads 100 scale divisions.

One unit of enzyme has been taken as that amount which would produce a color showing 50% transmission in 10 minutes.[3] Figure 1 shows curves from which the activity of a preparation may be read directly. This unit is approximately 26 times that used by Tilden and Hudson.[4]

[3] In the similar case of α-amylase, where it is also possible to follow the hydrolysis by the determination of reducing groups, this point represents the splitting of about 5% of the total glycosidic bonds present in the starch (14).

[4] It was frequently convenient to use smaller volumes of the starch digestion mixture than 15 ml. When this was done, the same volume of the digestion mixture as before (i.e., 0.50 ml.) was diluted with iodine. As the unit described refers to the total enzyme quantity present in a 15 ml. system, the result with a smaller volume must be multiplied by the appropriate factor.

While macerans amylase is stable in dilute iodine solutions, its activity is relatively slow and does not appreciably influence the results of the procedure described here, provided the measurement of light transmission is made within a reasonable time.

The flatness of the curves in Fig. 1 at high light-transmission levels may be due to the formation of some iodine-Schardinger dextrin complex which is nearly colorless, at the expense of the blue iodine-starch complex.

Growth of Cultures. The authors are indebted to Dr. S. C. Husdon for cultures of *Aerobacillus macerans.* These were grown on oatmeal medium (15) in 2.8-l. Fernbach flasks. The contents of such a flask were used to inoculate a 5-gal. bottle of the same medium. A stream of air (roughly 2 l. per minute) was passed through the culture. The air was first sterilized by passage through sulfuric acid, and again humidified by passage through sterile water. The cultures were grown at 38°C. for 2 weeks, and in this time developed about twice the concentration of enzyme that was obtained from the still cultures in the flasks.

Samples removed at intervals were centrifuged and assayed. The result of one (typical) experiment is shown in Fig. 2. Practically all the enzyme found came into solution in this instance between the 11th and 13th day, and thereafter remained constant for 12 days more.

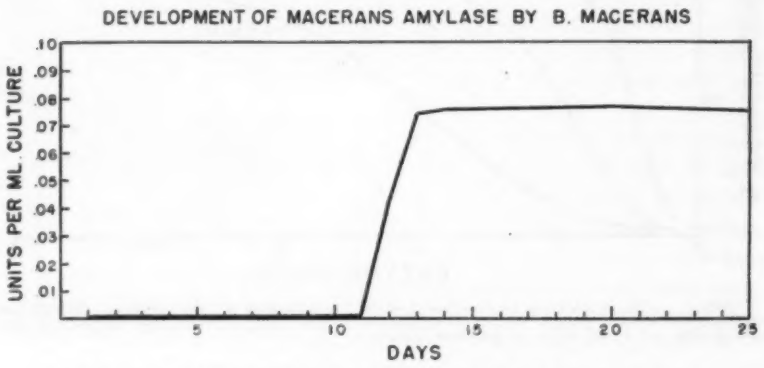

FIG. 2. Day by day development of macerans amylase by *Bacillus macerans* grown on a 5% oatmeal medium in 5-gallon bottles at 39°C.

Tilden and Hudson (15) noticed the same phenomenon and attributed it to autolysis of the bacteria. However, our attempts to obtain more enzyme from the large mass of cellular matter remaining in the medium by treatment with ethyl acetate, toluene, lysozyme, papain, or crude trypsin did not meet with success.

Purification of the Enzyme. Even when the enzyme content of the culture medium has reached its maximum, it is too dilute to give a precipitate with ammonium sulfate. It was necessary to filter the medium (with Filter Cel) and concentrate the clear liquid about 5-fold by evaporation below 40°. There was no loss of activity during this operation.

The concentrated medium was dialyzed against running tap water long enough to remove most of the calcium. It was then made 0.75

saturated with (solid) ammonium sulfate. After standing overnight at room temperature, the precipitate was filtered off, dissolved in as little water as convenient, and reprecipitated at 0.6 saturation. The precipitate was again dissolved in as small a quantity of water as practicable and dialyzed against tap water to remove salts. The enzyme was next precipitated from this solution (working at about −10°C.) by the addition of an equal volume of acetone; the precipitate was centrifuged out and redissolved in water so that each milliliter contained about 20–25 units. The pH was adjusted to 5.5 with $M/5$ HCl and (working at 0–5°) twice the volume of 66% by volume ethyl alcohol was added. After the addition of alcohol the solution could be brought to room temperature without any marked loss of activity. Any precipitate which formed thereafter in an hour at room temperature was centrifuged out and discarded. The enzyme was then ready for adsorption on starch.

An adsorption column was used containing equal weights of commerical wheat starch and Celite. About 20 gm. of the adsorbent was employed for each 35–50 ml. of the alcoholic enzyme solution, which was sucked through the column under reduced pressure. The column was washed with dilute alcohol (44% by volume) until the washings were colorless, and then eluted with water containing salts designed to simulate the tap water of this locality.[5] As the last portion of enzyme is eluted rather slowly from the column, the volume of eluate may be inconveniently large. The enzyme was therefore concentrated in vacuum as before (below 40°) without loss of activity. This step had the added advantage of removing traces of alcohol. The concentrated solution yielded a white precipitate at 0.6 saturation with ammonium sulfate (the solid salt was added directly). The specific activity of this precipitate (units of macerans amylase/mg. protein nitrogen) was 54–58, compared with 7 in the culture fluid and 9–10 for the material just prior to adsorption. These data do not show the relatively great amount of non-protein material removed from the preparation. They do indicate perhaps that macerans amylase represents a fairly high proportion of the total protein in the original culture fluid.

The overall yield of the purification process here described has varied from 25 to 45% of the enzyme originally present; at best about 5 mg. of protein per liter of culture fluid that contained roughly 6 gm. of total solids after dialysis.

[5] For a long time the only satisfactory eluant of the enzyme appeared to be tap water, and numerous attempts to replace this with buffers at the same pH gave poor results. Finally, in order to describe a method that was independent of the local water supply, a synthetic imitation thereof was tried with excellent results.

The composition of the solution was as follows in terms of mg per liter of distilled water: $Na_2SiO_3 \cdot 5H_2O$, 42 mg.; $FeCl_3 \cdot 6H_2O$, 9.66 mg.; $AlCl_3 \cdot 6H_2O$, 26.8 mg.; $CaSO_4 \cdot 2H_2O$, 48 mg.; $MgSO_4 \cdot 7H_2O$, 50.7 mg.; K_2CO_3, 5.28 mg.; $NaHCO_3$, 62.5 mg.; $CuSO_4 \cdot 5H_2O$, 0.39 mg., 0.1 N NaOH to bring pH to 9.0.

Results and Discussion

Probable Unity of Macerans Amylase. At the outset of this work there seemed to be a fair probability that the "macerans effect" was the result of more than one enzyme. Furthermore it was apparent that the presence of an enzyme of the α-amylase type would invalidate the assay method used until it was removed. There is now much better evidence for the (still unproved) assumption that the formation of Schardinger dextrins from starch may be attributed to a single enzyme.

Calibration curves for the assay method as illustrated in Fig. 1 also have been made with the purified enzyme, and compared with those originally prepared from concentrated culture fluid. For practical purposes the two sets of curves are superimposable. This indicates that nowhere during this extensive purification process has the removal of part of a necessary complex of enzymes taken place. Furthermore, periodic examination throughout the digestion of starch by macerans amylase has shown that the crystals of the dextrin-iodine complexes appear of the same two shapes despite the purity of the enzyme used. Massive red-brown crystals of hexagonal cross-section appear first; these are later replaced, though never entirely, by long yellow needles. Tests with the separated dextrins have shown that only the needles are given by purified β-dextrin. The course of the reaction thus appears to be unchanged by purification.

The resistance of macerans amylase to dilute iodine is evidence that enzymes resembling α and β-amylase (in this respect) are not included in the "macerans effect." Thus 4.54 units of purified macerans amylase in 0.003 *N* iodine were kept at room temperature for three and one-half hours. The iodine color was then discharged with thiosulfate solution—4.30 units of macerans were found in the system. After

TABLE I

COMPARING THE FORMATION OF SCHARDINGER DEXTRINS BY PURIFIED MACERANS AMYLASE FROM STARCH AND FROM DIGESTION PRODUCTS OF STARCH MADE WITH α-AMYLASE

Substrate	Enzyme	Crude Mixed Dextrins gm.	2 X[3] Dextrin gm.	3 X[3] β-Dextrin	
				gm.	$[\alpha]_D^{25}$
Potato starch 20 gm.	Crude	10.6	5.3	4.5	155°±4°
Potato starch 20 gm.	Purified	14.2	5.7	4.3	155°±4°
α-Amylase dextrin 1.9 gm.	Purified	1.90	1.30	1.10	159°±4°
α-Amylase dextrin 15 gm.[1]	Purified	5.8	3.2	—	155°±4°[2]

[1] Digestion in the presence of trichloroethylene for 3 days at room temperature. These conditions are regarded as optimum for the production of the α-dextrin (8).
[2] On 2 X crystallized material.
[3] X represents times crystallized.

TABLE II

REDUCING SUBSTANCES FORMED FROM STARCH BY MACERANS AMYLASE AND MALT α-AMYLASE

Time	*Milliequivalents per ml. of digestion mixture (calculated as maltose)*	
	Macerans amylase	*Crystalline malt α-amylase*
1.5 min.	0.0004	—
10 min.	.0000	0.0063
15 min.	.0008	.0098
20 min.	.0015	.0104
30 min.	.0008	.0137
40 min.	.0004	.0149
50 min.	.0004	.0168
60 min.	—	.0181
2 hrs.	.0006	—
3 hrs.	.0003	—
24 hrs.	.0021	—
Complete hydrolysis of the starch to maltose (calculated)	0.041	0.041

22 hours, 4.47 units were recovered. Similar treatment would have destroyed the amylases of malt, barley, or sweet potatoes. Macerans amylase was also found to be stable when treated with periodic acid, despite the formation of formaldehyde in the system. Both malt and sweet potato amylases lose their activity under similar conditions.

After elution from starch, the enzyme preparation was found to produce no significant amounts of reducing substances from starch in 24 hours. Table II shows the results obtained when reducing substances were measured by the dinitrosalicylic acid method (1) in a system prepared as described for the assay: 1.33% Lintner soluble starch, containing enzyme and 0.068 *M* calcium acetate buffer, pH 5.2, incubated at 40°. When purified macerans amylase was used in a concentration of 0.033 unit per ml. of digestion mixture, no appreciable amount of reducing sugars was found in 24 hours, although this amount of enzyme was sufficient to bring the starch to the achromic stage (with iodine) in 60 minutes. However, 44% of the same substrate was digested (calculating the product as maltose) in 60 minutes by crystalline malt α-amylase (0.017 unit per ml.), and 61% was digested in 10 minutes by a heavy dose (ca. 15 units per ml.) of crystalline sweet potato β-amylase.

The foregoing is in contrast with the behavior of culture fluids of macerans, as reported by Kneen and Beckord (9), who observed a substantial production of fermentable material, particularly after 20–24 hours incubation. The existence of a second enzyme may be postu-

lated as originating either in yeast or in macerans and concerned with the breakdown of Schardinger dextrins, but not necessarily with their formation.

Action of Macerans Amylase on Dextrin Formed by α-Amylase. Macerans amylase was found to form appreciable quantities of Schardinger dextrins from starch which had already been extensively hydrolyzed by malt α-amylase. A dextrin was prepared from freshly dissolved Lintner starch in 5% solution, by digestion with crystalline malt α-amylase at room temperature until a sample of the solution no longer gave any appreciable color with iodine. Four volumes of 95% ethanol were then poured into the digest and a few drops of saturated sodium chloride solution were added to aid coagulation of the precipitate, which settled completely overnight. The alcohol was then poured off, the precipitate dissolved in water, and the solution boiled for 5 minutes. Thereafter it was filtered through Celite and reprecipitated with alcohol as before. The reprecipitated dextrin was dried in vacuum to constant weight. Its reducing action as determined by Jansen and MacDonnell's modification of the Willstätter-Schudel method (8) was 0.316 milliequivalent of reducing groups per gram of dextrin, corresponding to an average chain length of about 19 glucose units. Table I compares the results obtained when this dextrin and potato starch were treated with macerans amylase.[6]

The substrates, used in the amounts indicated, were digested with 0.6 unit of macerans amylase per gram for about 20 hours at 40°. The mixed dextrins were precipitated with trichloroethylene as directed by McClenahan, Tilden and Hudson (10). Their procedure was also used for isolating the β-Schardinger dextrin from the mixture, except that the solutions were made about twice as dilute. The α-Schardinger dextrin fractions were sticky and isolation of the α-Schardinger dextrin in well defined crystalline form was not satisfactory. However, the α-Schardinger fractions gave heavy crops of the typical hexagonal crystals of the iodine complex, and very few of the needle-shaped crystals which are typical of the β-Schardinger dextrin. On the other hand, all specimens of the recrystallized β-Schardinger dextrin gave nothing but needle-shaped crystals with iodine. The yields have been calculated as though all the crude dextrin obtained in each case was used for the isolation of β-Schardinger dextrin, though in fact only 3–5 gm. of each sample was actually used for this purpose.

Effect of Maltose, Glucose, and β-Schardinger Dextrin on the Rate of Action of Macerans Amylase. When purified macerans amylase

[6] A similar experiment was made in which the limit dextrin was prepared by the digestion of soluble starch with crystalline sweet potato β-amylase. Macerans amylase also formed Schardinger dextrins from this substrate as evidenced by typical needle and hexagonal crystals of the Schardinger complexes with iodine. However, the α-dextrin seemed much more predominant. The crystalline dextrins isolated with trichloroethylene were low in yield, and have not been studied further.

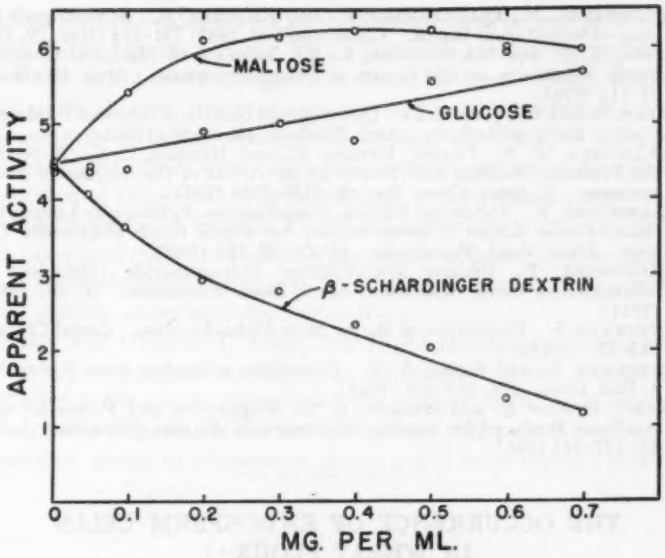

FIG. 3. Effect of maltose, glucose, and beta-Schardinger dextrin on the rate of action of macerans amylase.

(specific activity ca. 55) was allowed to act upon Lintner starch, as described in the assay method but also in the presence of β-Schardinger dextrin, it was found that the dextrin was inhibitory (Fig. 3). This is not surprising, for it is not unusual that an end product may be inhibitory to further enzyme action. It was also observed, however, that both maltose and glucose accelerated the disappearance of starch, maltose being much more effective (Fig. 3). This observation is in accord with French *et al.* (4) that Schardinger dextrins may form straight-chain bodies by adding other sugar residues. The composition of such digests is being investigated.

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THE OCCURRENCE OF ENDOSPERM CELLS IN WHEAT FLOUR [1,2]

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ABSTRACT

The identity of certain particles of flour with the endosperm cells of the wheat grain is confirmed by establishing similar dimensions (about 40 × 200 μ). Molybdenum blue in aqueous solution stains the endosperm cell walls but not the gluten; this enables the cell wall to be seen on the surface of particles. Whilst the walls are readily stripped from the cells, partly or completely, during milling, the cell contents may persist as organized units. The use of the stain reveals that loose folds or separate sheets of the cell wall tissue occur abundantly in all flours.

Observations on Holdfast (hard) and Scandia (soft) English grown wheats confirm the finding of previous workers that whilst in soft wheat the cells break up during milling, in hard wheats the fractures tend to occur between the cells. In hard wheat the extent to which individual cells separate is affected by the moisture content of the wheat.

It is well known that the endosperm of the wheat grain consists of thin-walled cells containing a protein matrix in which starch granules are embedded. Alexandrov and Alexandrova (1) described these cells with particular reference to their content of starch granules, which vary characteristically in different parts of the grain, and Sandstedt (4) discussed their formation in the developing grain. Berliner and Rüter

[1] Manuscript received April 14, 1950.
[2] Contribution from The Research Association of British Flour-Millers, St. Albans, England.

(2) described the various types of endosperm cells occurring in German-grown wheat grains. They also examined laboratory milled flours sifted through a sieve of aperture size 0.143 mm. They found that flour particles from German-grown soft wheat were loosely cohering agglomerates of small disrupted particles, showing no trace of cell structure, but flour from Manitoba wheat contained a substantial proportion of larger cell fragments, said to be typically the prismatic cells which make up most of the Manitoba grain. In soft wheats the low strength of the cell contents allows them to break up during milling, but in hard wheats the fractures tend rather to occur between the cells, which tumble apart into discrete units.

Greer and Hinton (3) found that slight moistening of thin sections cut from vitreous grains caused the appearance of fine cracks. With hard wheats the cracks reproduced the pattern of the endosperm cells, but with soft wheats they ran indiscriminately through the cell contents. If portions, either of vitreous or mealy grain, were pressed while being

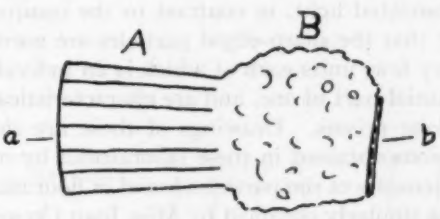

FIG. 1. A particle of semolina (sizings) showing ridged and starchy structures. For explanation of lettering, see text.

viewed under low-power magnification, the endosperm of hard wheats was seen to break along the lines of the cell walls, producing particles in the form of whole cells or multiples of cells. On the other hand, with similar treatment, the endosperm of soft wheats broke down in random fashion, the cells being disrupted. It was suggested that these observations, which supported the conclusions of Berliner and Rüter, might be useful to plant breeders, since they could be applied to individual grains.

The cells have now been recognized during microscopical examination of milling stocks. Thus, in semolina, particles are seen of the type shown diagrammatically in Fig. 1. Under low-power magnification, portion A is ridged and waxy in appearance; B is softer and more starchy; *a* shows the imprint of the (detached) aleurone layer; *b* is a piece of branny tissue from the crease. A is a bundle of intact cells; the exterior surface of B represents broken cells, whose starch interior is exposed.

Such recognition is subjective but is convincing to an observer of the cells both *in situ* in the grain and in fragments of milling stocks. The demonstration by Berliner and Rüter of the occurrence of endosperm cells in some flours was of this nature, depending on subjective appreciation of varying shapes and sizes of particles in flour. No numerical data for sizes were given.

Identification of Flour Fragments

It is desirable in the first place to collect as much evidence as possible showing the identity of the characteristic fragments in flour with the cells in the grain. Evidence so far obtained in these laboratories may be grouped under the following headings:

1. *Shape and Size of Units.* In ordinary flour, examination of the air-dry material at 30-fold magnification shows a mixture of sharp-edged particles having the appearance of crystals, with loose, opaque material. The sharp-edged particles are translucent, almost transparent, to transmitted light, in contrast to the opaque particles. It has been found that the sharp-edged particles are normally composed of one, or a very few, units each of which is an individual endosperm cell or a substantial part of one, and are characteristically in the form of needles or long prisms. Drawings of these are shown in Fig. 2. Air-dry dimensions obtained in these laboratories by means of microscopical measurements of the particles found in flour may be compared with dimensions similarly obtained by Miss Joan Crewe of cells *in situ* in Manitoba grain which were exposed in a midway transverse section.

	Length μ	*Width* μ
Flour particles		
Single cells or parts of cells	150–250	35– 60
Pairs of cells	220–300	64–120
Endosperm cells *in situ*		
Dorsal portion: needle like cells	>200	40– 56
Near crease: needle like cells	128–200	40– 64
Center of cheek: large polygonal cells	120–144	80–120
Center of cheek: smaller rounded cells	72–104	64– 96

Inspection of the above data is helped by reference to Fig. 3 which is a drawing of the cross section made by Miss Crewe.

Individual needle-like cells carefully dissected from near the dorsal side of air-dry Manitoba grain were found to be approximately 200 μ in length and 40–50 μ in width when measured without moistening.

When flour containing intact cells was separated into ranges of particle sizes by means of thorough sifting with a nest of closely graded sieves, the particles in the coarser fractions, e.g., through No. 12, over No. 14 silk, were largely present as double or triple bundles of cells,

whilst those in the finest fraction, through No. 20 silk, as individual units. The lengths of mesh aperture of Nos. 12, 14, and 20 silks are 115, 95, and 65 μ, respectively.

Considerable swelling of the cells occurs immediately on contact with water and it is proportionately much greater in the direction of

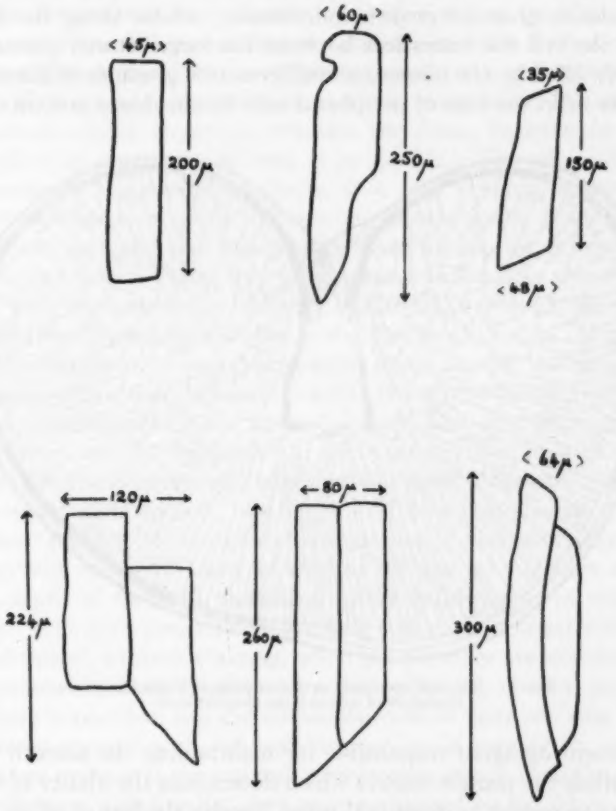

FIG. 2. Typical sharp-edged particles found in flour. *Upper row:* single cells; *lower row:* duple cells. Dimensions in microns.

the width than along the length of the cells. Some preliminary measurements indicate that it may be 20% in length and 50–100% in width. Cells with intact covering appear to swell in width less than naked cell contents. These observations relate to uncovered specimens. Even light pressure by the cover slip distorts the moistened cells; again the distortion is greater in the direction of the width than in that of the length of the cells.

2. *The Nature of the Surface and the Physical Behaviour of the Units.* Whether covered by its cell wall or not, the contents of each individual cell is recognizable as an intact entity by the nature of its outline. Under magnification of 100–200 diameters this is characteristically hard and straight whereas that of broken cell contents is wavy and indeterminate. The difference lies in the fact that in the latter case the larger starch granules project individually, whilst along the natural side of the cell the interstices between the larger starch granules are smoothly filled by the numerous smaller starch granules of about 1–2 μ diameter or in the case of peripheral cells by the dense protein matrix.

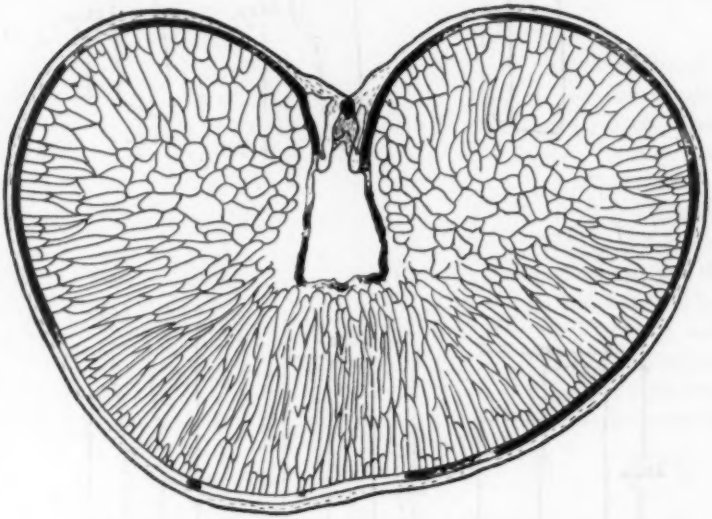

FIG. 3. Mid-way transverse section of grain of Manitoba wheat. Shaded band indicates the aleurone layer.

The cementing agent responsible for maintaining the smooth outline is doubtless the protein matrix which determines the ability of the cell contents to persist as organized units, despite the loss of all or part of the cell wall.

With gentle to-and-fro manipulation of the cover slip starch dispersal and gluten formation begin immediately in the case of broken or disorganized flour particles but much less readily in that of the units under discussion, which display elasticity and withstand relatively much manipulation before being broken up, irrespective of whether the cell wall is intact or not.

The number of the larger starch granules liberated from most of the individual endosperm cells varies from 20–35. This is very roughly

the order that might be expected from considerations of dimensions. It must, however, be remembered that the small proportion of relatively small endosperm cells whose greatest dimension does not exceed 60 μ and which are situated immediately next to the aleurone layer usually contain only starch granules of size intermediate between the large and small granules found in the more central cells.

3. *The Appearance and Manner of Occurrence of the Cell Wall.* Berliner and Rüter (2) stated that the endosperm cells, each enclosed in a thin cellulose membrane, are firmly joined by a cementing substance. We have no evidence however of the existence of this cement as a separate visible structure although the tissue between two adjacent cells may sometimes be seen to be double. The cell wall may be conveniently examined by means of a 3% aqueous solution of colloidal molybdenum oxide, known as molybdenum blue.[3] This stains the cell wall, and also any damaged starch granules, or "ghosts," present in the flour. Congo red in aqueous solution also stains both "ghosts" and cell wall but, in addition, heavily stains the gluten. The usefulness of molybdenum blue lies in the fact that it leaves the gluten practically unstained, so that it is possible to see the cell wall in superficial view as a blue sheet where it overlies the otherwise colorless contents of the endosperm cell. Points of particular interest found by using this stain are: (a) In bundles of cells the molybdenum blue stains the cell wall *in situ* between the individual units and it appears as a deep blue line when seen "end-on," see Fig. 4. If such particles are caused to roll over slightly, by suitable manipulation of the cover-slip, the thin deep blue line may often be seen to become a blue sheet of decreasing depth of tint and increasing width as the angle of rotation increases. The appearance of the dividing wall is geometrically similar to that obtained, without staining, when the particles are examined in a non-aqueous liquid of suitable refractive index, e.g., benzyl alcohol.

b) More often than not the individual cells in flour are seen to be incompletely covered with cell wall or even to be completely naked. Often tatters or folds of the cell wall may be seen attached to the edges of the otherwise naked cells; they may be caused to unfold or wave about by slip manipulation in a manner unmistakably characteristic of delicate itssue. Separate sheets or flakes of the tissue may frequently be seen, with the aid of the stain, amongst the flour particles.

c) In units where the cell wall substantially remains *in situ*, the edge of the blue sheet is seen in superficial view to be exactly parallel with the sharp outline of the cell contents, see Fig. 5.

d) If the units are disintegrated through movement of the cover slip in the way described, the previously attached pieces of cell wall

[3] Molybdenum Oxide Blue, British Drug Houses Ltd., London.

Fig. 4: Sheet of cell wall, seen end on as a deep blue line running longitudinally through the particle.

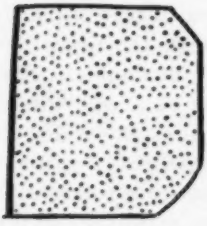

Fig. 5: Sheet of cell wall co-terminous with particle. Hard outlines under higher power are shown here in bold outline.

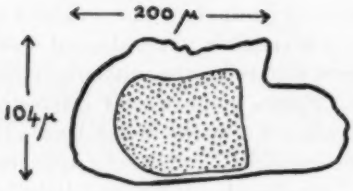

Fig. 6: Cell wall attached to a disorganized particlr having soft outline.

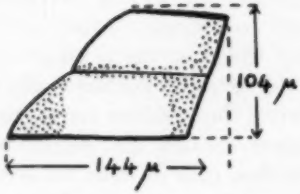

Fig. 7: *Left:* a "plate"; *right:* a "cube" or short rectangular prism.

FIGS. 4–7. Various types of endosperm particles found in flour. Dotted areas indicate cell wall as stained by Molybdenum blue.

are left plainly visible as blue sheets. These are of varying size and shape, but they frequently reach a length of 200 μ already given as characteristic of the needle-shaped endosperm cells. Sometimes the sheets show deep-stained longitudinal lines or ridges, i.e., the rows of lateral attachment of adjoining cells.

e) The amount of cell wall, shown by disintegrating the particles in the presence of the blue stain, is surprisingly great, both in British 85% extraction flour and in high-grade patent flour. The amount of

cell wall associated with particles excavated from the central endosperm appears to be as great as that with the cells near the periphery.

General Discussion

The foregoing observations lead to the following summary of the main types of particles appearing in flour:

Group I. Disorganized endosperm particles. These may be of varied structure, e.g., they may be flakelets, where the entire cell has been crushed and burst, or they may be robust portions of a cell whose surface is formed by the exposed cell contents. Provisionally, the category is taken to include all particles the major part of whose boundary as seen in projection on the microscope slide is formed of projecting large starch granules. Small particles and detached starch granules are not included in making a count.

Group II. Organized cells, or cell contents, the major part of whose outline is hard, or sharp-edged. This group includes:

A. Cells recognized by the shape and character of contained starch granules as originating from the central endosperm. These cells, characteristically, are rounded in shape.
B. Cells similarly recognized as of peripheral origin. These also are usually rounded but considerably smaller than the central cells.
C. Cells of prismatic or needle-like shape. These may originate from various parts of the grain.

Each of these groups may include particles consisting of one, two, or more cells. Particles also occur, consisting of cells of each of groups A and C, or B and C. The cell units may be either intact cells or parts of cells.

Finally in each sub-group of Group II the cell units may be classified as:

i. Substantially covered with cell wall as shown by the moylbdenum blue stain,
ii. Partly covered,
iii. Substantially uncovered.

It should be added that disorganized particles may be larger than any of the individual cell units. Frequently they are attached to pieces of cell wall, some of which may be large, as illustrated in Fig. 6.

Particles composed of prismatic cells may appear in various shapes. The make-up and dimensions of examples of such particles are drawn in Fig. 7. The shaded or colored areas show the parts covered with cell

wall. Such particles are brightly translucent and often difficult to distinguish in casual examination under low magnification from particles of glass or mineral substances.

Practical Application

1. *Flours from Hard and Soft Wheat Types.* Reference has already been made to the fact that the endosperm of soft wheats tends to break up indiscriminately whilst that of hard wheats tends to break up into fragments consisting of one or more cells. This has been found to apply to the English-grown wheats, Scandia and Holdfast, which represent, respectively, soft and hard types. Flours milled in the laboratory from Scandia wheat, and from vitreous and starchy Holdfast samples were examined.

With both samples of Holdfast the flours contained a large proportion of organized cell contents; the Scandia flour on the other hand contained mostly disorganized endosperm particles. A striking difference was that even in the finest fraction, separated from the flour by sifting, the Scandia particles were associated individually with much larger amounts of torn but loosely adherent cell wall than were the Holdfast.

These observations support the views, cited in the introduction, of Berliner and Rüter on the break-up of wheat endosperm. The present work also leads, through the staining application of molybdenum blue, to the recognition that the persistence of the cell contents as organized units is independent of the cell wall itself. The cell wall is clearly delicate, being readily shed or torn off. Alexandrov and Alexandrova (1) reported that the endosperm cells of hard wheats have thicker cell membranes and separate from the mass of endosperm more easily than

TABLE I

EFFECT OF MOISTURE CONTENT OF MANITOBA WHEAT ON PERCENTAGE FREQUENCY OF MULTIPLE, DUPLE, AND SINGLE ENDOSPERM CELLS IN FLOUR [1]

Wheat Moisture Content	Total Number of Particles Counted	Percentage Frequency			
		Multiple Cells	Duple Cells	Single Cells	
				Intact	Fragmentary and Disorganized
%		%	%	%	%
12.3	348	19.3	18.4	31.9	30.4
16.4	294	8.2	21.4	30.3	40.1

[1] A statistical analysis showed that the frequency of occurrence of multiple cells was significantly smaller in the flour milled from wheat conditioned to 16.4% moisture content. χ^2 calculated on the frequency distribution of 642 particles (which for convenience of presentation has been converted in the above table to percentage frequency) was 18.98; $n = 3$; significant at 0.001 level.

those of soft wheats during the preparation of microscopical sections. So far our own evidence does not show that hard wheat cells have thicker or stronger cell walls than soft.

2. *Effect of Conditioning.* Although differences described between hard and soft wheats may be regarded as well established, the effect with any one type of varying condition of the wheat has not been reported. The ease of separation of the cells during milling is considerably affected by the moisture content of the wheat. For example, Table I shows the effect of milling No. 1 Manitoba wheat at its natural moisture content of 12.3%, and after moistening to 16.4% and allowing to lie 17 hours before milling. Both tests were made under similar laboratory milling conditions and the flour extraction obtained in each case was in the range 74–76% of the original wheat.

It is well known by millers that drier milling tends to give a more granular flour. The data in Table I show clearly how with this wheat the difference in granularity essentially depends on the greater proportion of units consisting of more than two cells in the case of dry milling, whilst the production of disorganized particles and fragments of cells is increased by one-third in the more moist sample.

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AUTOLYSIS OF FLOUR AS AN INDEX TO ALPHA-AMYLASE ACTIVITY[1]

C. M. HOLLENBECK[2] and WENDELL REEDER[3]

ABSTRACT

The decrease in the swelling power of wheat starch in alkali upon the autolytic digestion of wheat flour dispersions at pH 5.7 for one hour at 65°C. in the presence of calcium ions was found to be an index of flour alpha-amylase activity.

This autolytic method appears promising for the control of malt supplementation of flour.

The addition of diastatically active malt supplements to flour and dough is now common practice in milling and baking. Several in-

[1] Presented at the annual meeting, May, 1950. Manuscript received June 30, 1950.
[2] Present address, Merck & Co., Inc., Rahway, New Jersey.
[3] This investigation was conducted in the laboratories of Campbell Taggart Research Corp., Kansas City, Mo., Present address, Dallas, Texas.

vestigators, including Sandstedt, Jolitz and Blish (6), Kneen and Sandstedt (2), Geddes (1), and Selman and Sumner (7), have discussed the function of malt amylases in baking. Such effects of malt supplementation in bread dough as improved dough handling, larger loaf volume, and better crumb structure are attributed to the action of alpha-amylase on the starch in the dough.

The optimum level of malt alpha-amylase in dough for the production of quality bread varies with the formula, procedure, and other factors. Obviously, it is desirable to use only the optimum level of malt in doughs, since excessive levels of malt are costly, and often result in bread with a gummy, dark-colored crumb. It is necessary then for the miller and baker to control carefully the amount of malt added to flour and this control necessitates a convenient, rapid and accurate method for measuring alpha-amylase in flour.

It is generally agreed that viscosimetric procedures are well adapted for use in controlling malt levels in flours and Selman and Sumner (7) have recommended the amylograph for this purpose. However, recording viscosimeters are costly and viscosity determinations are time consuming. Consequently, there is need for a simple method which requires only ordinary laboratory equipment and minimum expenditure of time. Such a simple, rapid method, based on the decrease in the swelling power of starch as the result of amylolysis, is described in this paper. The method which is patterned after those of Molin (5) and Kent-Jones and Amos (4), measures the action of alpha-amylase on flour starch during autolytic digestion of flour suspensions at elevated temperatures.

Materials and Method

Reagents. (1) Buffer solution. A stock concentrated aqueous mixed acetate buffer solution is prepared containing 40 g. of anhydrous sodium acetate, 40 g. of calcium acetate monohydrate, and 4 ml. of glacial acetic acid per 500 ml. The diluted buffer for the flour suspensions is prepared by diluting 25 ml. of this concentrated solution to 1,000 ml. with water. (2) Sodium hydroxide solution, approximately 11 *N*. The concentrated sodium hydroxide solution used for the neutralization of sulfuric acid digests in the Kjeldahl Nitrogen procedure may be employed if the concentration is at least 10 *N*.

Determination. Weigh duplicate 1.5 g. samples of flour, one for the blank and the other for the autolytic digestion. Disperse the flour sample in 50 ml. of dilute buffer solution (at room temperature) contained in a 125 ml. Erlenmeyer flask by stirring with a glass stirring rod for about 1 minute. (Leave the stirring rod in the flask.) Place the flasks in a water bath maintained at 65°C. and add, with stirring,

50 ml. of dilute buffer solution which has been preheated to 65°C.[4]. To the blank, add immediately, with stirring, 2 ml. of the concentrated sodium hydroxide solution. Pour a 40 ml. portion of the hot, alkaline suspension into a graduated, conical centrifuge tube and cool in a cold water bath to about 20°C. Digest the other sample, which is used for the measurement of autolytic amylase activity, for one hour at 65°C., taking the time from the moment the flask is placed in the bath. Stir occasionally during digestion. Add alkali,[5] transfer to a conical centrifuge tube and cool as described for the blank. Centrifuge both suspensions at a standard selected speed and time. (In these studies the suspensions were centrifuged in a Size 1, Type S.B., International Centrifuge for 4 minutes at Resistor Setting of 25.) Stop the centrifuge by slight application of the brake and record the volume of flour solids (Sediment Volume, S.V.) in each tube. The sediment volume ratio (S.V.R.) is calculated by the following formula:

$$\text{S.V.R.} = \frac{\text{volume of flour solids (sample)}}{\text{volume of flour solids (blank)}}$$

Results

Samples of "unmalted" flour from four different mills were experimentally supplemented with increasing increments of malted wheat flour and the S.V.R. values determined. The results are shown in Table I.

TABLE I

VARIATION OF SEDIMENT VOLUME RATIO WITH MALT ADDITION

Flour Sample Number	Sediment Volume Ratio			
	0.1% Malt	0.2% Malt	0.3% Malt	0.6% Malt
1	0.70	0.65	0.58	0.36
2	0.70	0.65	0.50	0.42
3	0.70	0.60	0.55	0.43
4	0.70	0.60	0.55	0.43

The straight line relationship between the S.V.R. values and the malt increments (Table I) validates the S.V.R. as a measure of alpha-amylase activity under the conditions of the experiment.

The S.V.R. values were determined on about 300 samples of bakers' patent flour representing the products of several mills throughout the Southwestern Area. Most of the flour samples were supplemented

[4] Several samples (the number depending upon the available equipment, etc). may be started at 3 minute intervals.

[5] It was noted that the degree of degradation of the flour starch during the autolytic digestion could be followed in a rough manner by the color produced on the addition of the alkali. The sugars and dextrins produced by alpha-amylase action were converted into yellow-colored compounds by the alkali. The higher levels of malt produced sufficient dextrins and sugars to give highly colored alkaline solutions.

with malt to a level for optimum bakery performance as determined by both amylograph viscosities and experimental baking. Based on the results with these flour samples, the range of S.V.R. values for optimum bake shop performance was from 0.3 to 0.5. The corresponding amylograph viscosity range, determined by a procedure similar to that described by Selman and Sumner (5), with the particular instrument used at the time was 160 to 240 units.

A scatter diagram of S.V.R. values and amylograph viscosities on 23 flour samples, picked at random, is shown in Fig. 1.

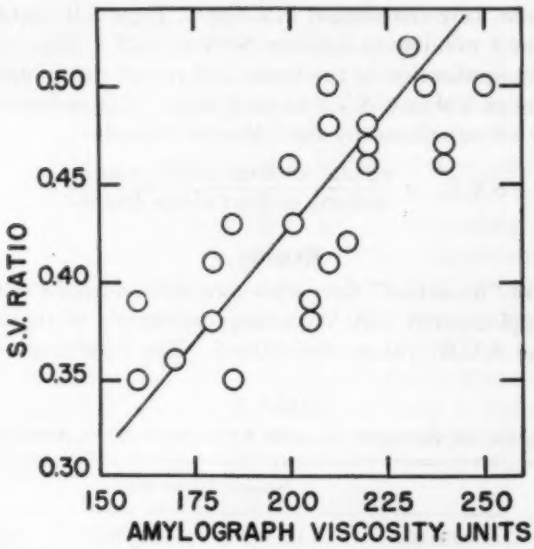

FIG. 1. The relation between sediment volume ratio and the amylograph viscosity of malt-supplemented flours.

The coefficient of correlation between amylograph viscosities and S.V.R. values on 50 samples of malt supplemented flour, picked at random, was 0.65, which is significant.

Discussion

The conditions for the sample digestion were selected as a compromise between the conditions required for high susceptibility of starch to enzyme attack and those at which alpha-amylase is stable. At 65°C. wheat starch is quite susceptible to amylase action, and according to Kneen, Sandstedt and Hollenbeck (3), a digestion temperature of 65°C. in the presence of calcium ions should not appreciably affect alpha-amylase activity.

The results of these preliminary studies indicate that the sediment volume ratio of a sample of flour is an index of the potential amylolysis of the flour starch by the alpha-amylase in the flour. Since the action of alpha-amylase on starch is the important reason for supplementing flour with malt, the S.V.R. value is an index to the "malt response" of a flour in terms of dough handling and loaf volume. Too, the method has possible merit for determining sprout damage in wheat.

With a possible sacrifice in accuracy and reproducibility, the method herein described offers an alternative to the recording viscosimeter as a method of measuring malt levels in flour. The chief advantages of the sediment volume method are speed and simplicity. By spacing samples at 3 minute intervals, 20 determinations or 10 samples can be handled with ease in about 1.5 hours, with only the ordinary laboratory equipment.

Because of the several variables which influence the S.V.R. value of flour, each laboratory interested in using this test should standardize the procedure to simulate, if not duplicate, the conditions herein described. Obviously, there is a limit to the applicability of the method in terms of potential alpha-amylase activity, since the linear relationship between S.V.R. values and malt increments can be expected only at relatively low levels of malt, i.e., at the levels normally used in baking.

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PURIFICATION OF FUNGAL MALTASE [1]

D. K. Roy [2] and L. A. Underkofler

ABSTRACT

A dry maltase concentrate has been prepared using filtrate from submerged culture of *Aspergillus niger* NRRL 330. Solid oxalic acid was dissolved in the filtrate, and then precipitated as calcium oxalate which carried down the maltase. The enzyme was eluted from the precipitate by means of phosphate buffer at pH 7.2, and then adsorbed from the eluate by means of Fuller's earth at pH 4.6. The maltase was again eluted from the solid residue by means of phosphate buffer, and precipitated from the clear eluate by adding cold absolute alcohol. The dry product was free of alpha-amylase and proteolytic activity, showed a scarcely measurable trace of limit dextrinase activity and a high maltase activity.

Maltase is found widely distributed in nature in most plant and animal tissues. Certain yeasts, molds, and bacteria are good sources of maltase. The specific substrates are maltose and alpha-glucosides for a true maltase. Certain enzymes having maltase activity, that is causing hydrolysis of maltose, have been found to attack higher carbohydrates also. For example, French and Knapp (5) found that maltase from *Clostridium acetobutylicum* was not specific for maltose but also produced hydrolysis of starch, amyloheptaose, and limit dextrins. This enzyme had no hydrolytic action on certain simple alpha-glucosides, such as alpha-methyl glucoside. The maltase of *Aspergillus niger* NRRL 330, which was that employed in the present investigation, is also capable of hydrolyzing certain higher glucose polymers according to Corman and Langlykke (3).

The separation and purification of fungal maltase is rendered difficult because of its instability. It deteriorates rapidly when in contact with common acids, salts and alcohols, the amount of destruction being influenced by the concentration of the reagent, temperature and time of contact. Hence, methods commonly employed for precipitatation of enzymes such as salting out and alcohol precipitation are generally ineffective in concentrating this enzyme.

Other workers have attempted purification of maltase. Michaelis and Rona (7) partially separated yeast maltase and invertase by treating yeast autolyzate with kaolin, maltase being adsorbed and in-

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Contribution from the Chemistry Department and Industrial Science Research Institute, Iowa State College, Ames, Iowa.
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vertase not. Willstätter and Bamann (10) improved the separation by employing hydroxides of aluminum as adsorbent for the maltase. Feigenbaum (4) prepared maltase free from invertase activity from a commercial mold diastase. He destroyed the invertase by means of sodium hydrosulfite and concentrated the maltase by dialysis against water and than alcohol. Although this procedure resulted in an enzyme preparation containing maltase and free from invertase, the method would not separate maltase from other enzymes such as alpha-amylase, limit dextrinase and proteases.

Schwimmer (9) concentrated and partially purified maltase from taka-diastase and mold bran. These enzyme products were prepared with *Aspergillus oryzae*, and the maltase from this organism had quite different properties, apparently, from the maltase of the *A. niger* used in our work. Schwimmer's maltase was quite stable in contact with acids, aqueous alcohol and ammonium sulfate.

Lipps, Roy, Andreasen, Vernon and Kolachov (6) partially purified maltase from *A. niger* by adsorption on Fuller's earth. Their procedure eliminated all alpha-amylase activity but caused destruction of much of the original maltase and appreciable limit dextrinase activity was still retained in their purest preparation.

It was the purpose of the research reported in this paper to attempt preparation of a maltase concentrate from *A. niger* free from alpha-amylase and limit dextrinase.

Materials and Methods

Mold filtrate was prepared as required by submerged culture of the mold *Aspergillus niger* NRRL 330. This culture was chosen for the work since Corman and Langlykke (3) reported filtrates from this mold strain to be relatively low in amylase activity and to have the highest maltase activity of any culture tested. The mold was cultured for production of maltase by a modification of the method of Adams, Balankura, Andreasen, and Stark (1). The medium employed contained 2% ground yellow corn, 0.5% Difco yeast extract and 0.5% calcium carbonate in tap water. The medium was sterilized in one liter quantities in 2-liter Erlenmeyer flasks equipped with alundum aerators. Each flask was inoculated with 20 ml. of two-day culture of the mold which had been cultivated in 100 ml. of medium of the same composition in 250-ml. Erlenmeyer flasks. The medium after inoculation was incubated for three days at 30°C. with continuous aeration, compressed air from the air line being passed through a sterile active charcoal filter and then through sterile distilled water. After three days of incubation the cultures were filtered, this combined filtrate serving as starting material for the purification of maltase.

The Fuller's earth used in the adsorption procedure was purchased from Fisher Scientific Company. In the later steps of the purification water redistilled in glass apparatus was employed. The regular distilled water provided in the laboratory was of questionable purity.

Maltase activity was evaluated throughout the course of the investigation by the procedure of Corman and Langlykke (3), the activity being expressed as per cent hydrolysis of maltose to glucose in two hours at 30°C. Alpha-amylase was determined by the method of Sandstedt, Kneen, and Blish (8), the activity being expressed as "units,"

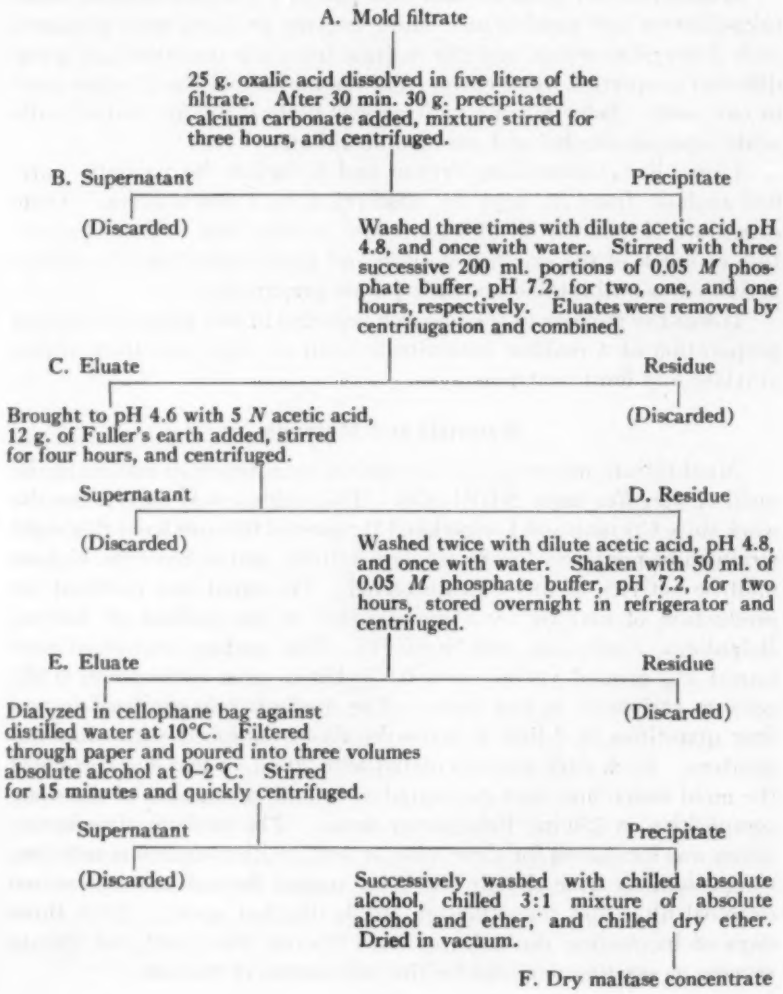

TABLE I

ENZYME ACTIVITIES OF FRACTIONS OBTAINED DURING SEPARATION AND CONCENTRATION OF MALTASE

Fraction	Alpha-Amylase, Units	Limit Dextrinase, Hydrolysis	Maltase, Hydrolysis
		%	%
A. Original mold filtrate	6.0	10.0	65.0
B. Filtrate after oxalate-calcium carbonate adsorption	5.0	7.0	10.2
C. Eluate from oxalate-calcium carbonate adsorption (diluted 5 times)	slight trace	4.2	75.5
E. Fuller's earth eluate (diluted 20 times)	0	trace	69.5
F. Dry enzyme concentrate (1.0 mg. sample)	0	slight trace	79.0

representing the number of grams of starch dextrinized in one hour at 30°C. The method of Back, Stark, and Scalf (2) was employed for determination of limit dextrinase, the activity being expressed as per cent hydrolysis of limit dextrin to fermentable sugar in one hour at 30°C. One ml. of enzyme solution was used in all determinations unless otherwise indicated.

Results

A number of procedures were tried for the concentration of maltase from the mold filtrate. These involved precipitations with various salts, with alcohol and acetone, and adsorption procedures. In most cases maltase was destroyed more rapidly than the other carbohydrases present. The most successful procedure is outlined on p. 74. This resulted in a final dry concentrate which had high maltase activity, was free of proteolytic and alpha-amylase activity, and had a slight trace only of limit dextrinase activity. The steps employed are presented in the flow diagram. The final solid maltase concentrate was dried *in vacuo* and stored in the refrigerator.

In Table I are given data on the enzyme activities of the materials at various steps of the procedure outlined in the flow sheet. The original mold filtrate had an alpha-amylase activity of 6.0 units per ml., one ml. produced 10.0% hydrolysis of limit dextrin, and one ml. caused 65.0% hydrolysis of maltose.

Much of the original alpha-amylase and limit dextrinase activities were retained in the discarded filtrate from the calcium oxalate adsorption. Losses of maltase during the concentration procedures were extensive. It was estimated that the total recovery of original maltase in the final precipitate was less than 10%. The major losses of maltase were in the last two steps.

The total yield of dry maltase concentrate was 280 mg. The analytical data shown on the last line of the table for this solid enzyme concentrate were obtained using one ml. samples of a 0.1% solution, corresponding to 1.0 mg. of the final dry enzyme concentrate. This amount produced 79.0% hydrolysis of maltose according to the procedure of Corman and Langlykke. No alpha-amylase activity could be found. A slight trace of limit dextrinase activity was detected, corresponding to less than 0.2% hydrolysis of limit dextrin by a 1.0 mg. sample of the concentrate. The precipitate showed no proteolytic activity, using gelatin and casein substrates.

Discussion

The main purpose of this investigation was to prepare a dry maltase concentrate free of other enzyme activities present in high degree in the original mold filtrate. Quantitative evaluation of the extent of purification of the maltase in terms of maltase units per mg. of solids or per mg. of protein in the various fractions was not attempted. Work is being continued on such a quantitative basis. Efforts are being made to improve the recoveries of maltase, and if possible to obtain the enzyme in crystalline form.

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COMMUNICATIONS TO THE EDITOR

On the "Dextrinase" Activity of Crystalline Amylase Preparations[1]

DEAR SIR:

It has been found that crystalline malt alpha-amylase (3) can hydrolyze a partially degraded dextrin mixture[2] obtained from the combined action of beta and bacterial amylases on starch (2). Thus Table I shows that approximately 40% of the linkages in the dextrin are ultimately hydrolyzable by crystalline malt alpha-amylase. However, the rate of hydrolysis by the enzyme is quite slow, as compared with its initial action on soluble starch. It has been calculated that the apparent initial rate of starch hydrolysis is roughly about 200 times that of the hydrolysis of this dextrin. The average chain length was changed from eight glucose units to two, corresponding to the hydrolysis of three of the seven linkages. However, when a dialyzed malt extract was allowed to act on the dextrin (the dose having the same alpha-amylase activity as used in the crystalline enzyme digest) approximately 60% of the linkages were hydrolyzed. The calculated average chain length was 1.6, corresponding to approximately four linkages split.

On the assumption that the substrate is a dextrin of eight glucose units, it is evident that glucose, although it may be formed here, is not necessarily an end product of alpha-amylase hydrolysis, whereas it *must* have been formed by the action of malt extract. This follows from consideration of the possible ways in which a chain of eight glucose units may be split. If three of the seven bonds are split (as with alpha-amylase), the greatest possible yield of glucose is three moles, but the smallest possible yield is zero. When, however, four of the seven bonds are split (malt extract), the maximum glucose yield is four moles and the minimum is two. Thus malt, but not alpha-amylase, must of necessity have some sort of true glucosidase action.

The initial rate of the hydrolysis of starch by the malt extract was about ten times that of the hydrolysis of the dextrin; and it may be seen that the first three linkages are hydrolyzed quite rapidly as compared with the hydrolysis of the fourth. Neither preparation contained maltase.

[1] Enzyme Research Division Contribution No. 131.

[2] The dextrin was obtained from the Wahl-Henius Institute, Chicago, Illinois, and is said to have been a "limit dextrin" prepared according to the directions of Dr. Eric Kneen. It is apparent, however, that it is not a true alpha-beta limit dextrin, since a criterion of purity for the latter is its resistance to even partially purified alpha-amylase.

TABLE I

HYDROLYSIS OF "LIMIT DEXTRIN"

Crystalline Alpha-Amylase				Malt Extract			
Time (hrs.)	$\frac{(C-A)}{(B-A)} \times 100$	$\frac{B}{C}$	$(C/A)-1$	Time (hrs.)	$\frac{(C-A)}{(B-A)} \times 100$	$\frac{B}{C}$	$(C/A)-1$
0.00	0.00	8.2	0.0	0.00	0.0	8.2	0.0
0.08	1.50	7.5	0.1	0.10	13.8	4.1	1.0
1.11	8.62	5.1	0.6	1.7	43.5	2.0	3.1
2.2	15.03	4.0	1.1	4.0	45.6	1.9	3.3
19.0	36.18	2.4	2.6	22.0	54.9	1.7	3.8
25.0	36.80	2.3	2.7	46.0	59.2	1.6	4.2
44.0	37.80	2.2	2.8				

The hydrolysis of a 2% limit dextrin solution at 30° (pH 4.75) was determined by comparing the reducing action (C) of the digest (by hypoiodite titration (3)) with that of the limit dextrin completely acid hydrolyzed (B). Representing A as the reducing value of the unhydrolyzed dextrin, $(C - A/B - A) \times 100$ is the per cent splitting of the dextrin. B/C is the average degree of polymerization of the digest and $(C/A) - 1$ is the number of linkages split per mole of dextrin (D.P. = 8.2). The digests contained 170 units of alpha-amylase per 30 ml. (corresponding to about 3 mg. of crystalline protein).

Thus it seems that malt must contain some additional factor of glucosidase activity, which can give rise to substantial amounts of glucose from a small dextrin.

Crystalline beta-amylase from sweet potato (1) was found (in fairly high concentration) to hydrolyze only 2% of the dextrin linkages in 48 hours under the same conditions as used for the alpha-amylase experiment.

SIGMUND SCHWIMMER
Enzyme Research Division
Bureau of Agricultural & Industrial Chemistry
Agricultural Research Administration
United States Department of Agriculture
Albany, California

September 6, 1950

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Determination of Moisture in Starch by Drying

DEAR SIR:

Reported investigations of procedures for determining moisture in starches by drying[1,2] generally have involved drying starch at various elevated temperatures in the presence or absence of air. Even with vacuum oven drying, variation in water content of 0.09%[1] resulted from changing the temperature from 80° to 100°C., and it was, therefore, questioned to what extent heat-produced decomposition affected the results. A quartz, spring-balance apparatus used in our studies of water sorption by starches permitted us to observe continuously the effect of heat and vacuum on samples of starch. It was found for corn, potato, and tapioca starches that samples dried at 25°C. to 10^{-6} mm. Hg pressure lost no more weight upon heating to 110°C. at this same pressure. Thus, there appears to be a very considerable range in temperature where truly constant weight can be achieved. It is realized that this does not prove that wet samples of starch dried at various temperatures will achieve the same constant dry weight; however, for those experiments[1,2] in which the same sample was dried at a series of temperatures, the variations in water content reported for drying in this temperature range undoubtedly result from the different relative vapor pressures of water effective in the methods rather than from starch decomposition.

N. N. HELLMAN
Northern Regional Research Laboratory,[3]
Peoria, Illinois

July 13, 1950

The Determination of Extraneous Matter in Non-Fat Dry Milk Solids

DEAR SIR:

Among the projects under study by the Committee of the New York Section on Procedures for the Examination of Food Products for Extraneous Materials has been a procedure for the determination of extraneous matter in non-fat dry milk solids. The following method has met with the acceptance of the Committee:

Place 100 g. of non-fat dry milk solids in a liter beaker, add 600 ml. of water and 30 ml. of conc. hydrochloric acid. Mix thoroughly and bring to a boil with constant stirring at the early stages to avoid char-

[1] Porter, W. L., and Willits, C. O. Moisture in potato starch. J. Assoc. Offic. Agr. Chemists 27: 179–194 (1944).
[2] Sair, L., and Fetzer, W. R. Determination of moisture in starch and its modifications. Ind. Eng. Chem., Anal. Ed. 14: 843–845 (1942).
[3] One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

ring. Add 20 ml. of light mineral oil and continue to boil gently for 20 to 30 minutes. After cooling, treat mineral oil layer in the usual manner. The separation is made with the separatory funnel as described by Walker[1]. Replace evaporation losses by washing down the sides of the beaker with water as required. If excessive bumping is encountered during the digestion period, this may be minimized by directing a stream of washed air through a glass tube into the bottom of the beaker.

This procedure is simply the general method worked out by the New York Committee applied to a specific problem. It permits the recovery and identification of insect fragments and rodent hairs which, on occasion, are found in non-fat dry milk solids.

EARL K. SPOTTS
Chairman 1949–50 Committee
Ward Baking Company
New York, N. Y.

ROBERT L. DOWDLE
Chairman 1950–51 Committee
Quality Bakers of America
New York, N. Y.

November 3, 1950

[1] Walker, N. H. Report of the 1945–46 Committee of the New York Section on Procedures for the Examination of Flour for Extraneous Materials. Cereal Chem. 24: 30–49, (1947).

Cereal Chemistry

EDITORIAL POLICY

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SUGGESTIONS TO AUTHORS

General. From January 1, 1948, an abstract will be printed at the beginning of each paper instead of a summary at the end, references will be numbered to provide the option of citing by number only, and date of receipt, author's connections, etc., will be shown in footnotes. Except on these points, authors will find the last volume of *Cereal Chemistry* a useful guide to acceptable arrangements and styling of papers. "On Writing Scientific Papers for Cereal Chemistry" (*Trans. Am. Assoc. Cereal Chem.* 6: 1–22. 1948) amplifies the following notes.

Authors should submit two copies of the manuscript, typed double spaced with wide margins on 8½ by 11 inch white paper, and all original drawings or photographs for figures. If possible, one set of photographs of figures should also be submitted. Originals can then be held to prevent damage, and the photographs can be sent to reviewers.

Titles and Footnotes. Titles should be specific, but should be kept short by deleting unnecessary words. The title footnote shows "Manuscript received . . ." and the name and address of the author's institution. Author footnotes, showing position and connections, are desirable although not obligatory.

Abstract. A concise abstract of about 200 words follows title and authors. It should state the principal results and conclusions, and should contain, largely by inference, adequate information on the scope and design of the investigation.

Literature. In general, only recent papers need be listed, and these can often be cited more advantageously throughout the text than in the introduction. Long introductory reviews should be avoided, especially when a recent review in another paper or in a monograph can be cited instead.

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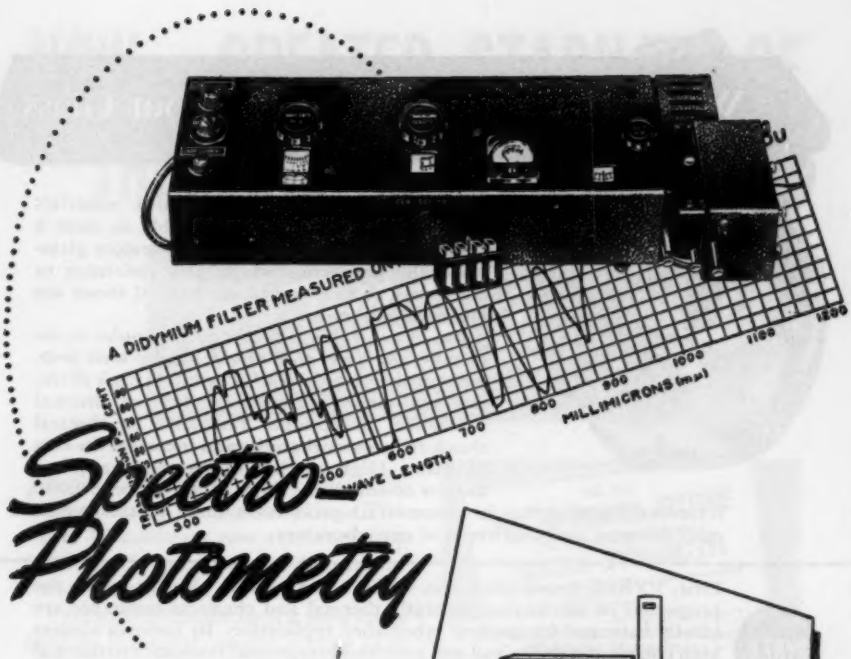

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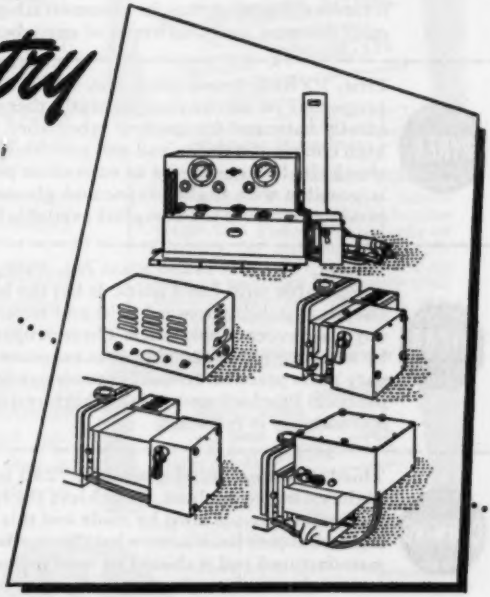

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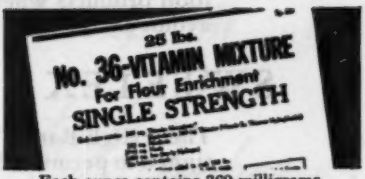

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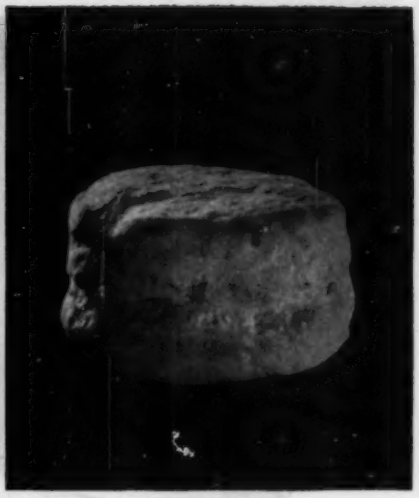

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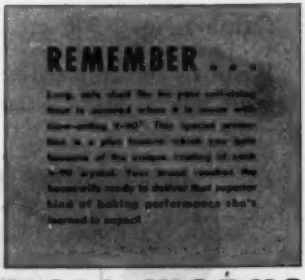

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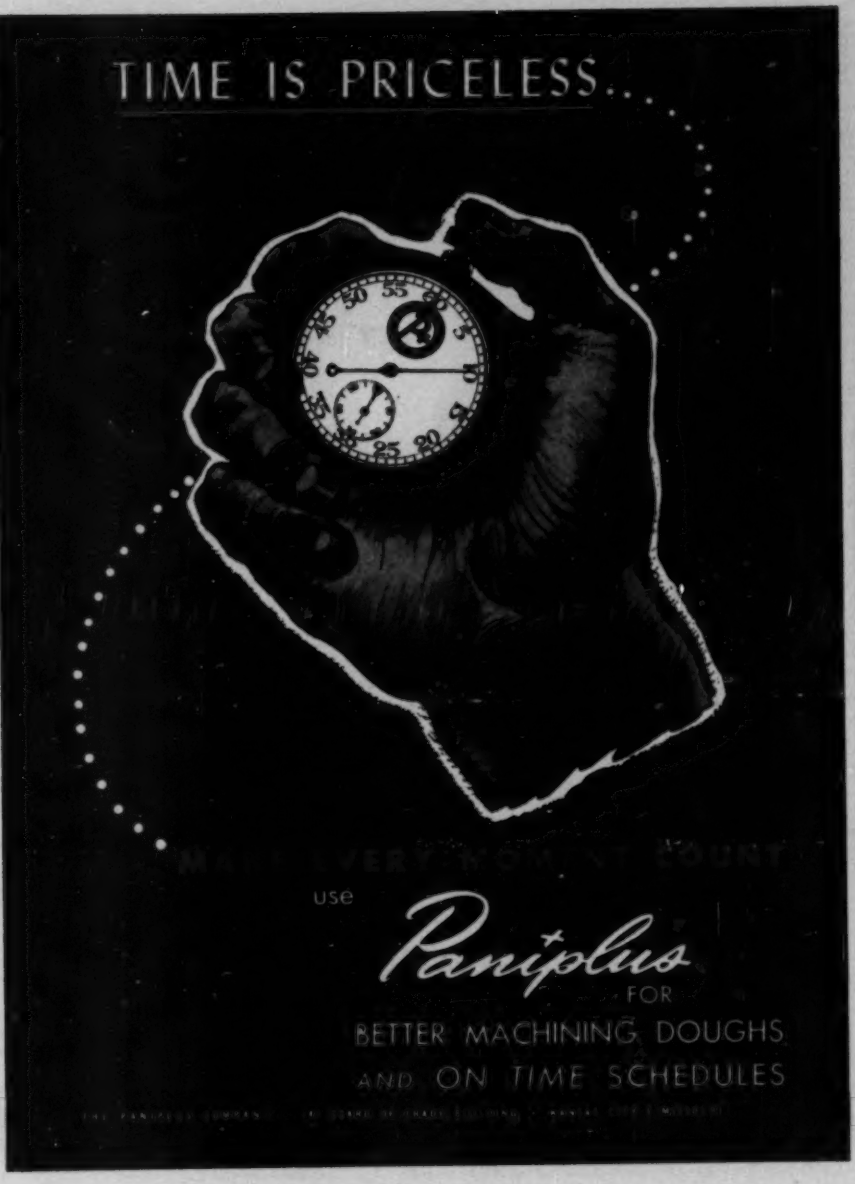
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